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Escherichia coli mastitis

Bacterial factors and host response

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Veterinary Medicine, University of Helsinki,
for public criticism in Auditorium Maximum, Hämeentie 57, Helsinki,
on 11 June 2004 at 12 noon

Helsinki 2004

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Cover. designed by Markus Pyörälä

ISBN 952-91-7340-7 (printed)
ISBN 952-10-1903-4 (PDF)
Yliopistopaino
Helsinki, 2004

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1. ABSTRACT

Mastitis caused by *Escherichia coli* is common in high-producing cows with a low milk somatic cell count. The severity and outcome of *E. coli* mastitis vary between cows of the same herd and between different lactation stages in the same individual. Variation in susceptibility of cows to *E. coli* mastitis and disease severity can be caused by differences in infecting bacteria or cows' immune response. Presence of some virulence factors has previously been reported in mastitis-causing *E. coli* bacteria, with serum resistance being the most important one. In early lactation, the decreased immune defence of the cow is regarded as the primary reason for increased susceptibility to *E. coli* mastitis. The aim of this thesis was to investigate bacterial and host factors affecting the severity and outcome of *E. coli* mastitis in dairy cows.

To study the bacterial factors in *E. coli* mastitis, 273 *E. coli* isolates of clinical bovine mastitis from Finland and Israel were studied by polymerase chain reaction to detect the genes for certain virulence factors. Serum resistance and capsule formation of the isolates were also examined, as these affect the pathogenicity of the strain. The studied isolates possessed a variety of different virulence factors, but none of them was common. The detected virulence factors, F17, S and P fimbriae, Afa8 afimbrial adhesin, cytotoxic necrotizing factor 1 and 2 (**CNF1** and **CNF2**), aerobactin and TraT, formed 29 different combinations, including different groups of serum resistance. The association between virulence factors and the severity of the mastitis in affected cows was evaluated in Finnish material; none of the virulence factors found from the isolates was associated with the severity of clinical signs. However, presence of genes for S and P fimbriae, CNF1 and CNF2 was significantly related to persistence of mastitis. This supports the earlier findings suggesting that chronic or recurrent *E. coli* mastitis could be caused by an udder-adapted strain of *E. coli*. The results indicate that to infect the bovine udder, specific virulence factors, e.g. those enhancing adhesion or invasion in the epithelia or damaging host cells, are not necessary for the *E. coli* bacteria and that the virulence factors of the infecting bacterial strain probably play a minor role in the severity of clinical signs of *E. coli* mastitis.

A total of 200 of the *E. coli* isolates were also tested for antimicrobial resistance. Resistance was low; 27% of the isolates were resistant to one or more antimicrobial agents and 11% were multiresistant. All but one multiresistance pattern included resistance to tetracycline, which is often related to resistance to other antimicrobials. We also found an association between resistance for some antimicrobials and presence of certain virulence factors. Tetracycline resistance was associated with presence of S and P fimbriae, CNF1, CNF2, aerobactin and TraT, resistance to ampicillin with aerobactin and resistance to dihydrostreptomycin with CNF2, F17 and aerobactin. These associations may cause selection of virulence factors or maintenance of antimicrobial resistance.

To study cow factors and host response, we used an experimental *E. coli* endotoxin model in which nine cows were challenged twice, in early lactation (**EL**) and in late lactation (**LL**). Cows showed a significantly more severe response in the EL than in the LL period. The difference was seen in systemic signs, whereas local signs were similar for both periods. The concentration of tumour necrosis alpha (**TNFα**) in milk increased after the endotoxin challenge, reaching higher levels in EL cows. No TNFα was detected in blood. Although TNFα might not be directly responsible for the systemic signs in endotoxin mastitis, it probably has a critical role in initiating host response by inducing the local production of other cytokines, which in turn mediate the systemic effects of the host response. TNFα also seems to induce production of serum amyloid A (**SAA**) in milk, as milk SAA concentration was higher in EL than in LL, and the concentrations of TNFα and SAA were

closely related at the cow level. In blood, the SAA increased later, after endotoxin challenge, with the average concentration being higher in LL, indicating differences in production and regulation of local and systemic SAA.

After induction of endotoxin mastitis, the number of polymorphonuclear leucocytes (**PMN**) in milk and blood increased faster, reaching higher levels in the EL than in the LL period. However, the function of blood neutrophils after endotoxin challenge, measured as chemiluminescence (**CL**), increased in LL but decreased in EL. This impaired function of blood PMN in EL could be caused by subclinical ketosis of the cow, reflected in the increased serum free fatty acid concentrations. The CL of milk PMN increased in both EL and LL, being more pronounced in EL and appearing simultaneously with the decreased CL of PMN in blood. This may have resulted from an influx of the most active PMN from the circulation to the milk, and from the stimulation of the milk PMN by such locally produced cytokines as TNFα. The function of PMN may though be more critical for host defence and for susceptibility of cows to *E. coli* mastitis than their actual number in blood and milk.

The results of this thesis supports the findings of previous studies showing that the increased susceptibility of the cow to *E. coli* mastitis and the more severe course of disease in early lactation depends more on host factors than on the characteristics of the infecting bacterial strain. The virulence and antimicrobial resistance of mastitis-causing *E. coli* reflect the situation of bacteria found in the environment or in the intestines or faeces of the cow. To prevent *E. coli* mastitis, efforts should be focused on improving the cows' environment and herd management.

2. LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers referred to in the text by their Roman numerals:

I. Kaipainen, T., Pohjanvirta, T., Shpigel, N.Y., Shwimmer, A., Pyörälä, S. and Pelkonen, S. 2002. Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. Vet. Microb. 85:37-46.

II. Lehtolainen, T., Shpigel, N.Y., Shwimmer, A., Honkanen-Buzalski, T. and Pyörälä, S. 2003. *In vitro* antimicrobial susceptibility of *Escherichia* coli isolates from clinical bovine mastitis in Finland and Israel. J. Dairy Sci. 86:3927-3932.

III. Lehtolainen, T., Pohjanvirta, T., Pyörälä, S. and Pelkonen, S. 2003. Association between the virulence factors and clinical course of *Escherichia coli* mastitis. Brief Communication. Acta Vet. Scand. 44:203-205.

IV. Lehtolainen, T., Suominen, S., Kutila, T. and Pyörälä, S. 2003. Effect of intramammary *Escherichia coli* endotoxin in early- vs. late-lactating dairy cows. J. Dairy Sci. 86:2327-2333.

V. Lehtolainen, T., Røntved, C.M. and Pyörälä, S. 2004. Serum amyloid A and TNF2α in serum and milk during experimental endotoxin mastitis. Vet. Res. In Print.

3. ABBREVIATIONS

AER	aerobactin
APP	acute-phase proteins
ASAT	serum aspartate aminotransferase
AU	arbitrary units
CL	chemiluminescence
CNF1	cytotoxic necrotizing factor 1
CNF2	cytotoxic necrotizing factor 2
DHS	dihydrostreptomycin
EGTA-BS	bovine serum with inactivated classical complement pathway
EL	early lactation
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acids
I-BS	inactivated bovine serum
IL	interleukin
LBP	LPS-binding protein
LL	late lactation
LPS	lipopolysaccharide
LT	heat-labile enterotoxin
mCD14	membrane CD14
MIC	minimal inhibitory concentration
MIC_{50}	minimal inhibition concentration for 50% of isolates tested
MIC_{90}	minimal inhibition concentration for 90% of isolates tested
MMP	matrix metalloproteinase
MXP	multiplex PCR
NAGase	N-acetyl-β-D-glucosaminidase
N-BS	normal bovine serum
PC	post-challenge
PCR	polymerase chain reaction
PCV	packed cell volume
PMN	polymorphonuclear leucocytes
PP	post-partum
ROS	reactive oxygen species
SAA	serum amyloid A
SCC	somatic cell count
sCD14	soluble CD14
ST	heat-stable enterotoxin
TNFα	tumour necrosis factor alpha
TS	trimethoprim-sulphadiazine
TSA	trypticase-soy agar
WBC	white blood cell

4. INTRODUCTION

Environmental mastitis caused by *Escherichia coli* has increased in many countries and herds at the same time as contagious mastitis has been successfully controlled (Lam, 1996b; Peeler et al., 2002). The proportion of *E. coli* as a causative agent in bovine clinical mastitis varies between countries. In Finland less than 20% (Pyörälä and Pyörälä, 1998; Nevala et al., 2004), whereas in Israel more than 60% (Shpigel et al., 1998) of clinical mastitis is caused by coliforms. The severity of the disease also varies between countries; *E. coli* mastitis is seldom very serious or fatal in Finland, whereas in Israel up to 16% of coliform mastitis cases result in the death or culling of the cow (Shpigel et al., 1994).

E. coli infects the udder via the teat canal (Eberhart, 1984). An environment contaminated with faeces is the main source of mastitis-causing *E. coli* bacteria (Linton et al., 1979; Nemeth et al., 1994). Environmental factors, herd management and use of antimicrobial agents may select bacterial strains and affect their virulence factors and antimicrobial resistance, causing the variation in the course of *E. coli* mastitis seen between countries and herds. Poor barn design and management of dairy cows may also provide the cows with predisposing factors for coliform mastitis (Ward et al., 2002; Krause et al., 2003).

The severity and outcome of *E. coli* mastitis can vary between cows in the same herd and in the same individual during different lactation stages. The observed systemic and local clinical signs result from the acute-phase reaction, which is the response of the host to any tissue injury caused by trauma or inflammation (Kushner, 1984; van Miert, 1991). The acute phase reaction differs depending on the physical condition of the individual. Physiological stress around parturition and metabolic diseases, for instance, may be associated with increased susceptibility of periparturient cows to coliform mastitis (Suriyasathaporn et al., 2000; Burvenich et al., 2003). Studies on spontaneous or experimental *E. coli* mastitis have shown that affected cows can be divided into mild or severe responders based on clinical signs and disease outcome (Pyörälä et al., 1994; Hirvonen et al., 1999; Wenz et al., 2001a). Periparturient and EL cows most frequently show severe clinical signs and even fatal outcome (Hill et al., 1979; Pyörälä and Pyörälä, 1998; Menzies et al., 2000).

In field cases, the onset of mastitis is unknown and the time of detection may affect the definition of severity. On a practical level, the most important issues are prevention, treatment and outcome of mastitis. It is generally agreed that the treatment of *E. coli* mastitis may be adjusted according to the severity of disease; only severely affected or immunosuppressed cows require aggressive treatment, including antimicrobial agents. It is important for the veterinary practitioner or the herd manager to be able to identify these cases based on severity of clinical signs and to be aware of the link between clinical signs and *E. coli* mastitis outcome.

5. REVIEW OF THE LITERATURE

5.1. CLINICAL CHARACTERISTICS AND OUTCOME OF *E. COLI* MASTITIS

Despite the numerous studies carried out on mastitis caused by *Escherichia coli*, many open questions remain concerning the pathogenesis and clinical course of the disease. When studying the aspects affecting severity of *E. coli* mastitis, the first problem encountered centres around the definition of the term severity; it can be based on clinical signs or outcome of mastitis (Pyörälä et al., 1994; Shpigel et al., 1994; Dosogne et al., 1997; Hirvonen et al., 1999; Wenz et al., 2001a). The intensity of clinical signs reflects host response at the time of observation, and varies during the course of mastitis. The final outcome, as rapid elimination of bacteria, prolonged infection or death of the cow due to endotoxin shock, describes more the ability of the cow to limit deleterious inflammatory reactions and to clear the infection (Burvenich et al., 2003). The host response may be more aggressive because of a particularly virulent bacterial strain invading the udder or a vast number of bacteria, which results in large amounts of released endotoxin. Host response may also be intense in a case of a more alert immune system. The outcome may become serious or fatal if the cow's immune system can not kill the bacteria due to their high virulence or excessive number, or an impairment of the immune system. Serious consequences may also occur if the cow's immune system is not suppressed after the bacteria have been cleared from the udder.

Different methods have been introduced for classifying the clinical course of *E. coli* mastitis. Cows can be divided into severe or mild responders based on intensity of general and local clinical signs, changes in appearance of milk and loss of milk production during mastitis (Pyörälä et al., 1994; Dosogne et al., 1997; Hirvonen et al., 1999; Wenz et al., 2001a). Cows can also be grouped by outcome or the ability to return to production (Shpigel et al., 1994). In experimental mastitis, the severity of some clinical signs, such as heart rate and rumen motility, have been shown to correlate with a high number of bacteria in the milk and with loss of milk production (Lohuis et al., 1990b).

The clinical characteristics and outcome of *E. coli* mastitis vary from mild mastitis, where cows have only local signs in the udder and the duration of the infection is short, to very severe or even fatal forms (Burvenich et al., 2003). *E. coli* mastitis typically has a sudden onset, which leads to changes in milk appearance, first to serous and yellow, and later to clotty and thick. Milk somatic cell count (**SCC**) increases to very high numbers. The udder becomes hard, swollen and tender. The cow also has systemic signs, generally including high fever, increased pulse frequency, reduced rumen contractions, lack of appetite, depression and decreased milk production. Studies using experimental *E. coli* mastitis models have shown that the first signs are usually noticed at the local level, at approximately 8 h post-challenge (**PC**), and fever and other systemic signs peak at 12 h PC (Lohuis et al., 1990b; Hirvonen et al., 1999; Hoeben, 1999; Haddad et al., 2001). In mild or moderate cases, the systemic signs vanish within 48 h and local signs within 7 days (Lohuis et al., 1990b; Pyörälä et al., 1994; Hirvonen et al., 1999). In severe cases, the cow may not survive or systemic signs may be prolonged, with milk production being lost permanently (Shpigel et al., 1998; Hirvonen et al., 1999; Rantala et al., 2002).

In experimental *E. coli* mastitis, bacteria can be detected in the milk until 5-7 days after inoculation; endotoxin is simultaneously detected (Pyörälä et al., 1994; Dosogne et al., 2002). However, a large variation exists in the duration of infection between experimental studies due to the different bacterial strains and doses used in the challenge. Bacteraemia or endotoxaemia occur seldom and only in very severe cases (Katholm and Andersen, 1992; Wenz et al., 1999; Wenz et al., 2001b; Dosogne et al., 2002). Bacteraemia was found significantly more often in cows with severe clinical

signs (Wenz et al., 2001b). Only trace amounts of endotoxin have been detected sporadically in the blood of cows with experimental *E. coli* mastitis (Dosogne et al., 2002). The release of inflammatory mediators, rather than the absorption of endotoxin, causes the systemic signs of *E. coli* mastitis (Verheijden et al., 1983; Lohuis et al., 1988; Burvenich et al., 2003).

5.2. *E. COLI* BACTERIA

E. coli is a Gram-negative, non-spore-forming rod, which belongs to the family *Enterobacteriaceae*. The cell wall of Gram-negative bacteria typically consists of three layers, the cytoplasmic membrane and the outer membrane, separated by a peptidoglycan layer (**Figure 1**). The outer cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (**LPS**). LPS comprises lipid-A, the lipopolysaccharide core and repeated polysaccharide units called O-antigens (**Figure 1**) (Cullor, 1996). Lipid-A is the lipophilic, inner part of LPS. The toxic effects of LPS also known as endotoxin, are caused by lipid-A (Cullor, 1996; Hogan and Smith, 2003). Here, the terms LPS and endotoxin are used synonymously. On the outer surface, bacteria may have fimbrias which protrude from the cell wall. The surface may be covered with a thick polysaccharide layer called a capsule. Based on the different antigenic structures of O-antigens, K-antigens (capsular) and H-antigens (flagellar), *E. coli* strains can be divided into O:H:K serotypes (Cullor, 1996).

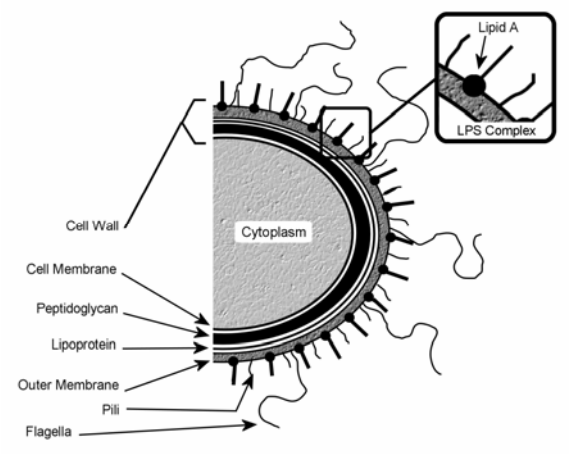


Figure 1. Schematic illustration of the components of Gram-negative bacteria (Redrawn from Cullor, 1996).

Escherichia coli is part of the normal intestinal flora of humans and animals. It is the most common facultative anaerobic bacterial species in the gut and is constantly excreted in the faeces to the environment. Pathogenic *E. coli* bacteria can cause intestinal and extraintestinal infections in mammalian and avian hosts (Cullor, 1996). Infections of the gastrointestinal tract may lead to various kinds of diarrhoeic diseases, which, in the case of shiga toxin, may even progress to systemic haemolytic ureamic syndrome in humans and oedema disease in pigs (Cullor, 1996). *E. coli* is the predominant cause of urinary tract infection in humans. *E. coli* also causes invasive diseases, such as bacteraemia and meningitis, in humans and animals. In avian species, *E. coli* is an important cause of respiratory and ovarian tract infections (Cullor, 1996).

5.2.1. Virulence factors

Pathogenic *E. coli* bacteria are typically specific to the type of disease they cause and to the animal species infected. They produce virulence factors involved in pathogenesis of specific diseases (China and Goffaux, 1999). Bacterial virulence factors are required to colonize and infect the host

and to fight host defence mechanisms. Major groups of *E. coli* virulence factors include toxins, adhesins, proteins secreted into host cells, polysaccharide capsules and O-antigens, and other mechanisms to resist killing by complement or to scavenge iron. Bacteria do not produce virulence factors constantly but only upon receiving certain signals from the host or environment (China and Goffaux, 1999). The genes for virulence factors may be present in the bacterial genome or may reside extrachromosomally on plasmids, even though the virulence factor is not produced (Harel and Martin, 1999).

5.2.1.1. Adhesins

Adhesins are either hair-like appendages known as fimbriae or pili, or afimbrial adhesins associated with the cell surface (Soto and Hultgren, 1999). Bacteria need adhesins to adhere to and colonize the host cell surface. Different adhesins have been detected in *E. coli* isolates associated with bovine diseases. The family of F17 fimbriae includes F17a expressed by bovine enterotoxigenic *E. coli*, F17b expressed by *E. coli* isolated from septicaemic and diarrhoeic calves and lambs, F17c isolated from septicaemic *E. coli* strains and F17d described in bovine enterotoxigenic *E. coli* and *E. coli* isolated from diarrhoeic calves (Le Bouguenec and Bertin, 1999). The F17 fimbriae are often associated with other virulence factors in pathogenic *E. coli*. Genes encoding F17 fimbriae are located on the chromosome (Bertin et al., 1996). In the study of Pohl and Mainil (1995), almost half of the F17-positive *E. coli* strains were resistant to complement and produced aerobactin.

S and P fimbriae are usually found in *E. coli* strains causing urinary tract infection (Soto and Hultgren, 1999). They are probably needed for adherence of bacteria to urinary tract epithelia and are suggested to be involved in recurrent urinary tract infections (Schilling et al., 2001). Genes encoding S and P fimbriae are located on the chromosome (Mainil et al., 1997).

Afimbrial adhesins are encoded by different *afa* genes; afa-7 and afa-8 have been found in bovine isolates, afa-8 being more common than afa-7 (Lalioui et al., 1999). Afa-8 genes have been detected on plasmids, which also carried genes for F17c and cytotoxic necrotizing factor 2 (**CNF2**) (le Bouguenec and Bertin, 1999). Another afimbrial adhesin found in bovine pathogenic *E. coli* strains is the capsule-like structure called CS31A adhesin (le Bouguenec and Bertin, 1999). It has been described in *E. coli* strains from calves with septicaemia or diarrhoea (Contrepois et al., 1986; Mercado et al., 2003). The genes encoding CS31A have been detected on large plasmids carrying genes for other virulence factors, such as aerobactin and F17c fimbriae, as well as for antimicrobial resistance (Contrepois et al., 1986).

5.2.1.2. Toxins

Pathogenic *E. coli* strains may produce a variety of toxins with different activities: heat-labile (**LT**) and heat-stable (**ST**) enterotoxins, shiga toxins and cytotoxic necrotizing factors 1 and 2 (**CNF1** and **CNF2**). Shiga toxins are produced by enterohaemorrhagic *E. coli* strains (Riley et al., 1983). Enterotoxigenic strains usually produce two enterotoxins, LT and ST, which have distinct roles in the pathogenesis. *E. coli* isolates producing CNF2 are common in cattle (Pohl et al., 1993). Many of these strains also produce F17b fimbriae and aerobactin and are resistant to the killing action of serum. The CNF1-positive strains are typically isolated from extraintestinal infections and are mainly positive for adhesins of the P and S fimbrial families or the Afa family (Mainil et al., 1997, 1999; Bertin et al., 1998). The production of CNF1 is chromosomally encoded, whereas CNF2 is encoded by genes located on a plasmid (Falbo et al., 1992; Mainil et al., 1999).

5.2.1.3. Aerobactin

Iron is an essential element for the survival and multiplication of *E. coli*. Aerobactin is one of the siderophores present in Gram-negative bacteria. It chelates iron, making it available for bacterial use. The gene for aerobactin is located either on a chromosome or on a plasmid, where many of the antimicrobial resistance genes are also located (Vidotto et al., 1991; Johnson et al., 2002).

5.2.1.4. Factors conferring serum resistance

Serum resistance is one of the most studied virulence properties of Gram-negative bacteria (**Table 1**). Resistance to the killing action of serum is linked to structures at or near the bacterial surface. Capsule or outer membrane proteins, like OmpA and TraT, have been suggested to interfere with complement components and the membrane attack complex, preventing the killing action (Ward and Sebunya, 1981; Taylor, 1983; Weiser and Gotschich, 1991; Pramoonjago et al., 1992).

TraT is a cell surface-exposed lipoprotein, which is assumed to inhibit the correct assembly or membrane insertion of the membrane attack complex of complement (Sukupolvi and O'Connors, 1990). TraT is encoded by the *traT* gene carried on large conjugative plasmids (Sukupolvi and O'Connors, 1990). The K1 capsular antigen protects *E. coli* from the killing effect of serum and has been thought to co-operate with TraT (Montenegro et al., 1985) However, in clinical studies (Nemeth et al., 1991; Wooley et al., 1993; Pfaff-McDonough et al., 2000), no relation of K1 to serum resistance or the *traT* gene could be detected.

5.2.2. Pathogenicity of *E. coli* isolated from mastitis

E. coli is an opportunistic bacterium when causing mastitis (Valente et al., 1988; Barrow, 1989; Nemeth et al., 1994). Bovine mastitis resembles urinary tract infection; the infection in both is ascending and caused by bacteria from the environment. The source of mastitis-causing *E. coli* strains may be found in the intestinal flora of the affected cow (Linton and Robinson, 1984), other cows or calves. Several different serotypes have been detected from the isolates causing mastitis, and *E. coli* mastitis is definitely not caused by a limited number of specific pathogenic strains (Linton et al., 1979; Linton and Robinson, 1984; Sanchez-Carlo et al., 1984b; Valente et al., 1988; Lipman et al., 1995; Jung, 1999). However, the existence of udder-adapted *E. coli* strains has been suggested, since the same serotype (Hill et al., 1979; Linton and Robinson, 1984) and genotype of *E. coli* have been isolated from cases of recurrent mastitis (Lipman et al., 1995; Lam et al., 1996a; Döpfer et al., 1999; Bradley and Green 2001). Isolates from recurrent mastitis have been shown to invade mammary epithelial cells *in vitro* faster and in larger numbers than the strains from occasional cases (Döpfer et al., 2000). Mastitis-causing *E. coli* strains may also have some common virulence factors to support their colonization, adherence and survival in the udder.

Serum resistance of *E. coli* has been studied since in the early days of mastitis research (Carroll and Jasper, 1977). In the previous studies, most of the isolates have been serum-resistant, although serum-sensitive strains have occasionally also been found (Sanchez-Carlo et al., 1984a; Valente et al., 1988; Barrow and Hill, 1989; Nemeth et al., 1991, 1994; Fang and Pyörälä, 1996). An experimental study by Carroll et al. (1973) showed that serum-resistant isolates caused longer and more severe *E. coli* mastitis than serum-sensitive strains, and serum-sensitive strains were even unable to cause clinical mastitis (Carroll et al., 1973). The lactation stage of the affected cow had no effect on serum resistance patterns of isolated organisms (Hogan et al., 1989).

Table 1. Prevalence of the previously studied virulence factors in *E. coli* isolated from mastitis.

Reference	No. of isolates	Virulence factor studied	Positive		Type of the study	
			No.	%	Geno-type	Pheno-type
Caroll and Jasper, 1977	?	Serum resistance		100		x
Wray et al., 1983	95	Haemagglutination	10			x
		Colicin V	3			x
	71	K-antigen	41			x
Sanchez-Carlo et al., 1984	184	Serum resistance	183	99,5		x
		ST	1			x
		LT	2			x
		Invasiveness	0			x
Valente et al., 1988	65	Serum resistance	65	100		x
		ST	0	0		x
		LT	0	0		x
Barrow and Hill, 1989	237	Serum resistance	230	98,7		x
		Haemagglutination	203	86		x
		Colicin	37	18		x
		Colicin V	10			x
		Vero toxin	1	0,5		x
		Capsule	172	75		x
Nemeth et al., 1991	95	Serum resistance	61	64		x
		TraT	41	43	x	
		K1	3	3		x
Pohl et al., 1993	29	CNF1	6		x	
		CNF2	1		x	
Nemeth et al., 1994	50	Serum resistance		76		x
		Aerobactin		20		x
		Colicin		14		x
		Colicin V		6		x
		Vero toxin		8		x
Lipman et al.,	20	F17	11	55	x	
		CNF1	1		x	
Burns et al., 1996	62	CNF1	1	1,6		x
		CNF2	0	0		x
Fang and Pyörälä, 1996	169	Serum resistance	102	68		x
Stephan and Kuhn, 1999	145	VT1	1		x	
		VT2	1		x	
		VT1 and VT2	2		x	
		eae	0		x	
		ehly	4		x	
		S and P fimbriae	2			x

Numerous virulence factors have been described among mastitis-causing *E. coli* isolates, but none of them has proven to be common (**Table 1**). The phenotypic methods used in most of the earlier studies might not always detect virulence factors since bacteria may produce them only under conditions in which they are necessary for bacterial survival.

5.2.3. Antimicrobial resistance

Broad-spectrum antimicrobial agents are generally used to treat coliform mastitis (Andersson, 1989; Erskine et al., 2002b), despite no convincing evidence exist of their benefit in treatment (Erskine et al., 1992; Pyörälä et al., 1994; Pyörälä and Pyörälä, 1998; Erskine, 2000). The use of antimicrobial agents causes selection pressure towards antimicrobial resistance among bacteria (Aarestrup, 1999; Prescott, 2000). Many of the genes encoding antimicrobial resistance are located in large conjugative plasmids (Falkow, 1975), and they may be linked together forming multiresistance (Levy et al., 1976; Prescott, 2000). The genes encoding the production of some virulence factors are also carried by plasmids and may be linked to the antimicrobial resistance genes in these plasmids (Hirsh et al., 1993). The antimicrobial resistance patterns of the bacterial population in the cows' environment can vary between countries or even between herds, reflecting the quantitative and qualitative aspects of antimicrobial treatments (Aarestrup, 1999; Anonymous 2001; Österbland et al., 2001). The use of antimicrobials may therefore also select bacteria with virulence factors linked to the antimicrobial resistance.

Antimicrobial resistance of *E. coli* isolates from bovine mastitis has been studied earlier by several authors (Bishop et al., 1980; Sogaard, 1982; Anderson, 1989; Trolldenier, 1995; Erskine et al., 2002b). Results have varied greatly, which may partly be due to the different methods and break-points used to determine susceptibility.

5.3. HOST RESPONSE

Host response is a complicated process in which the non-specific and specific immune systems act together to attack and destroy the invading pathogen and repair the damage the pathogen or the immune system itself has caused to the host (van Miert, 1995; Burvenich et al., 2003).

The first host defence against invading bacteria is the physiological barrier of the teat canal. Tight and rapid closure after milking and during the dry period prevents influx of bacteria. After invading the udder, the bacteria have to deal with humoral factors present in milk. During the dry period a high concentration of lactoferrin is one of the most effective factors against infections caused by coliform bacteria. Lactoferrin chelates iron and prevents growth of coliforms, which require large amounts of iron. In the lactating period, the concentration of lactoferrin in normal milk is not high enough to work efficiently, and the elevated citrate concentration further interferes with it (Kremer et al., 1990). During the middle of dry period the cow is thus quite resistant to clinical *E. coli* mastitis. However, in early and late dry period there may be acquisition of new *E. coli* infections, which become clinical during the first 100 days of lactation (Bradley and Green, 2000).

The bacteria killing ability of serum is thought to be an important defence mechanism against *E. coli*. The serum killing effect is based on complement and its ability to cause lysis of bacteria. The complement system also enhances PMN phagocytosis by attracting phagocytes, opsonizing bacteria and priming the intracellular killing of phagocytes (Rainard, 2003). The origin of complement components in bovine milk probably differs between normal and mastitic milk. In normal milk, the transudation of liver-produced complement proteins from the serum and the local synthesis of C3

may dominate (Rainard, 2003). Moreover, in this milk, the killing function of the complement may not be as important as previously thought since the amount of some complement compounds is low and other components of milk interfere with the complement (Kremer at al. 1990; Rainard, 2003). During inflammation the vascular permeability increases, causing exudation of complement proteins from serum, and the local synthesis by macrophages is simultaneously stimulated by proinflammatory cytokines (Rainard, 2003). This results in a different composition and a higher amount of complement proteins in milk, enhancing the bactericidical activity.

The most important defence mechanism against invading bacteria is the acute-phase response, which in *E. coli* mastitis is initiated by endotoxin released from bacteria (Olson et al., 1995). A great number of cytokines are produced by mammary macrophages, epithelial and endothelial cells and polymorphonuclear leucocytes (**PMN**) during the inflammatory process. The cytokines have various effects in initiating, mediating and reducing inflammation. In *E. coli* mastitis, the released endotoxin is bound by LPS-binding protein (**LBP**), and recognition of this complex is done by the membrane CD14 (**mCD14**) receptor of monocytes, macrophages and neutrophils, which are present in milk, causing the release of tumour necrosis factor alpha (**TNFα**) (Kielian and Blecha, 1995; Paape et al., 2003; Wang et al., 2003). The soluble CD14 (**sCD14**) may also bind endotoxin, either directly or by binding the endotoxin–LBP complex (Bannerman, et al., 2003; Paape et al., 2003; Wang et al., 2003). The endotoxin-LBP-sCD14 complex is recognized by the Toll-like receptors on the epithelial and endothelial cells of the mammary tissue, causing secretion of chemoattractants (Paape et al., 2003). Cytokines, interleukines (**IL**) and chemoattractants in turn act both locally and systemically, attracting neutrophils from the circulation to the infection site, opsonizing the pathogen and inducing production of acute phase-proteins (**APP**) in the liver (Hoeben et al., 2000a; Paape et al., 2003). After starting the acute-phase reaction, the speed of PMN efflux to milk and their ability to phagocytose the bacteria are critical to the development of mastitis (Burvenich et al., 1994). If the acute-phase reaction or bacteria killing ability of PMN is reduced, bacteria are able to multiply, and the damage caused to the host cells increases. On the other hand, an over-reaction in the acute phase may also cause damage so the inhibiting and repairing actions of the acute-phase reactions have to be in balance (Burvenich et al., 2003).

Differences in the production, function and kinetics of cytokines and APP may partly explain the variation in local and systemic signs of individual animals with *E. coli* mastitis (Hill et al., 1979; Pyörälä and Pyörälä, 1998; Hirvonen et al., 1999; Blum et al., 2000; Menzies et al., 2000; Ohtsuka et al., 2001).

5.3.1. Neutrophil function

In a healthy udder, the number of PMN in milk is low, < 100 000 cells/ml (Pyörälä, 2003). After any damage caused by bacteria or other pathogens or by physical injury, the number of PMN increases dramatically. This increase is mediated by a chemoattracting stimulus, and the degree of the stimulus depends on the infecting pathogen. In *E. coli* mastitis, a high number of PMN is required at the infection site immediately, and mature circulating PMN present in blood migrate towards the infection site, leading to neutropenia. If the chemoattracting stimulus continues, the reserve pool of mature PMN, located in blood vessels and in bone marrow, moves into the circulation and then to the infection site.

Immature PMN are seldom normally present in bovine blood (Paape et al., 2003). They are seen if neutropenia, caused by migration, is corrected by the release of immature PMN from the bone marrow. PMN are produced in the bone marrow by granulopoiesis. Maturity of PMN has an impact on their function (Dosogne, 1998; Mehrzad, 2002a; Burvenich et al., 2003); immature PMN have

impaired phagocytosis as well as decreased production of reactive oxygen species (**ROS**). Their migration to the infection site is also slower. Migration of PMN from blood circulation to the infection site depresses their functions; these cells have decreased phagocytosis because the migration utilizes the PMN energy sources. Ingestion of fat and other milk compounds also interferes with phagocytosis. The most important factor in intramammary defence against invading pathogens are the resident PMN (Burvenich et al., 1994).

PMN recognize the pathogen with the help of complements and other opsonizing factors. After recognition, they are able to use two different mechanisms for killing invading bacteria: oxidative-dependent and oxidative-independent mechanisms. The oxidative-independent mechanisms involve a variety of different enzymes and proteins in the non-azurophilic granules of PMN, such as lactoferrin, lysozyme, cytosine, elastase and fibronectin, which inhibit the growth of *E. coli* bacteria (Burvenich et al., 2003; Paape et al., 2003). Production of lactoferrin by PMN is, however, minimal compared with that by mammary tissue (Pfaffl et al., 2003).

A more effective mechanism against *E. coli* bacteria is, however, the oxygen-dependent mechanism. It takes place when the plasma membrane of the PMN recognizes the bacteria and releases superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in a respiratory burst (Mehrzad et al., 2002a; Paape et al., 2003). The oxygen-dependent mechanisms, which reside in the azurophilic granules of PMN, also involve the release of other ROS and nitrogen oxide after activation of myeloperoxidase during phagocytosis (Burvenich et al., 2003; Paape et al., 2003). ROS production of PMN can be measured by chemiluminescence (**CL**), which reflects the bactericidal capacity (Hoeben, 1999; Mehrzad et al., 2001a).

Low initial numbers of PMN, their immaturity and poor function in blood (Lohuis et al., 1990a; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997) as well as a low somatic cell count (**SCC**) in milk (Shuster et al., 1996) correlate negatively with the severity of induced *E. coli* mastitis.

5.3.2. Tumour necrosis factor alpha

TNFα belongs to the proinflammatory and inflammatory cytokines released from cells during inflammation (Shuster et al., 1996; Persson Waller et al., 1997). The role of TNFα in the pathogenesis of *E. coli* mastitis has been studied widely. It is supposed to have a pivotal role in initiating the effects of endotoxin during *E. coli* mastitis. TNFα also causes systemic effects, seen in changes in hormone secretion, milk production and composition, inflammatory parameters and clinical signs (Kushibiki et al., 2003). Some studies have found a correlation between severity of response and concentrations of TNFα in blood or milk (Hirvonen et al., 1999; Blum et al., 2000; Hisaeda et al., 2001; Ohtusaka et al., 2001), while other studies have reported no direct correlation (Nakajima et al., 1997; Hoeben et al., 2000a). TNFα also affects PMN functions by increasing the size of PMN and enhancing oxidative burst activity (Paape et al., 2003). However, if excessive quantities of TNFα are produced, numerous harmful effects will follow, including reduced blood pressure and tissue perfusion, intravascular thrombosis and severe metabolic disturbances, often leading to lethal shock (Bemelmans et al., 1996).

5.3.3. Serum amyloid A

In cattle, serum amyloid A (**SAA**) is a sensitive APP (Boosman et al., 1989; Alsemgeest et al., 1994; Hirvonen et al., 1999; Horadagoda et al, 1999; Heegaard et al., 2000; Karreman et al., 2000). It has also been shown to reflect the severity of mastitis (Eckersall et al., 2001). Cows with clinical mastitis have been reported to have increased levels of SAA in serum and milk compared with healthy cows (Eckersall et al., 2001; Pedersen et al., 2003); high levels have also been found in bovine colostrum (McDonald et al., 2001). SAA is mainly produced in the liver but also extrahepatically by macrophages, endothelial cells, mammary epithelial cells and smooth muscle cells (Jensen and Whitehead, 1998; Uhlar and Whitehead, 1999; Eckersall et al., 2001; McDonald et al., 2001). Extrahepatically produced SAA consist of multiple isoforms (Alsemgeest et al., 1995); M-SAA3 is present in normal milk and colostrum (McDonald et al., 2001). The effects of SAA may differ locally and systemically and may be concentration-dependent (Uhlar and Whitehead, 1999). Local and systemic production of SAA may also be governed by different control mechanisms (Eckersall et al., 2001).

5.3.4. Effect of age and lactation stage

The clinical response and outcome of *E. coli* mastitis vary according to the age of the animal and the stage of lactation (Hill et al., 1979; van Werven et al., 1997; Pyörälä and Pyörälä, 1998; Mehrzad et al., 2002b). Older cows are more susceptible to *E. coli* infections because of diminished PMN functions (Mehrzad et al., 2002b). It has been shown that *E. coli* intramammary infections can originate from the late dry period and become clinical in early lactation (Bradley and Green, 2000). Peracute *E. coli* mastitis with severe clinical signs most frequently occurs during the periparturient period and in early lactation (Hill et al., 1979; Pyörälä and Pyörälä, 1998; Menzies et al., 2000). This is thought to be due to the compromised immune mechanisms of the cows during the peripartum period (Mehrzad et al., 2001b).

A compromised immune system may result for several reasons. The numbers of circulating PMN in the blood change according to lactation stage; they increase at parturition but then decrease rapidly during the puerperal period, reaching a lower level than seen in later lactation (Kehrli et al., 1989; Zerbe et al., 2000; Kulberg et al., 2002). Cortisol levels may affect the number and function of PMN, and a positive correlation between PMN and cortisol has been detected (Preisler et al., 2000). However, cortisol may interfere with the number and efficient function of PMN (Hopster et al., 1998; Kulberg et al., 2002). In milk, cortisol increases at the time of parturition, but falls back to preparturition levels one day post-partum; in blood, no changes occur in cortisol levels (Schwalm and Tucker, 1978; Preisler et al., 2000).

During the post-partum period the diapedesis of PMN into the infected quarters (Hill et al., 1979; Hill, 1981) and phagocytosis (Kehrli et al., 1989; Hoeben et al., 2000b; Mehrzad et al., 2001b) are impaired compared with later lactation. PMN functions may be altered due to hyperketonaemia (Suriyasathaporn et al., 2000; Zerbe et al., 2000), which is commonly seen during early lactation (Jorritsma et al., 2001). Severe signs in experimentally induced *E. coli* mastitis were not associated with a mild negative energy balance, as indicated by elevated levels of non-esterified fatty acids or β-OH-butyrate (Kornalijnslijper et al., 2003), but an association was found with ketonaemia (Kremer et al., 1993).

The more severe response seen in early lactation may also be caused by a higher production of TNFα by mononuclear cells during this period (Sordillo et al., 1995). In endotoxin mastitis, TNFα was detected in plasma and milk of early-lactating cows (Blum et al., 2000; Hoeben et al., 2000a)

but not in mid-lactating cows (Shuster et al., 1993). However, in the last-mentioned study, the amount of endotoxin used for the induction of mastitis was much lower.

6. AIMS OF THE STUDY

Factors affecting the severity and outcome of *E. coli* mastitis in dairy cows were investigated in the thesis. These included both bacterial and cow factors. Specific objectives were as follows:

1. To characterize the virulence factors present in *E. coli* isolates from bovine mastitis.

2. To determine the antimicrobial susceptibility of *E. coli* isolates from bovine mastitis.

3. To investigate associations between both virulence factors and antimicrobial resistance of isolates and the clinical course and outcome of *E. coli* mastitis.

4. To investigate differences in response of early- and late-lactating dairy cows in the experimental *E. coli* endotoxin mastitis model.

5. To investigate the roles of TNFα and SAA in experimental *E. coli* endotoxin mastitis in early- and late-lactating dairy cows.

7. MATERIALS AND METHODS

This thesis comprises two main studies: a study of clinical mastitis caused by *E. coli* in Finland and Israel, and a study of experimental endotoxin mastitis.

7.1. FIELD DATA (I, II, III)

7.1.1. *Escherichia coli* isolates

A total of 273 *Escherichia coli* isolates (160 from Finland and 113 from Israel) from acute clinical cases of bovine mastitis were investigated. In Finland, the isolates were collected during 1990-1996 (16-34 isolates per year). In Israel, the isolates were collected during the years 1996-1997, 21 isolates in 1996 and 79 isolates in 1997. All isolates were studied for virulence factors (**I**), and 100 isolates from both Finland and Israel were randomly chosen for antimicrobial susceptibility testing (**II**). From the Finnish isolates, 155 were used to study the associations between virulence factors and clinical signs (**III**); the five excluded isolates were missing background information.

Milk samples were taken during routine farm calls by the attending veterinarian from the affected quarter of each cow using an aseptic technique. For bacterial isolation, 0.01 ml of milk was cultured as described by the National Mastitis Council (NMC, 1999). From the Finnish milk samples, the milk N-acetyl-β-D-glucosaminidase (**NAGase**) activity was determined. After 3 to 4 weeks, follow-up samples were taken from the Finnish cows for bacterial and milk NAGase analyses; 26 cases missed the resampling.

7.1.2. Conventional bacteriology

Isolates were preliminarily identified as *Escherichia coli* by phenotypic methods: colony morphology on a blood agar, Gram stain and growth on an EMB agar (NMC, 1999). The diagnosis was later confirmed using the API 20E test (bioMérieux, Marcy l'Etoile, France) (NCCLS, 1999). The isolates were stored in a semi-solid broth (Lab Lemco Broth, Oxoid, Hampshire, UK) at room temperature.

7.1.3. Isolate classification

From the Finnish material, clinical signs and changes in milk appearance were recorded at the time of sampling. Cases of mastitis were classified as mild or severe and persistent or non-persistent. In mild mastitis, cows had no or slight fever and only moderate changes in the udder and milk. In severe mastitis, cows were depressed, had high fever, no appetite, a swollen and painful udder and substantial changes in milk appearance. The intramammary infection was classified as persistent if the follow-up sample harboured *E. coli* growth. The cows were also divided into three groups according to lactation stage: 0-21, 22-210 and over 211 days post-partum (**PP**).

7.2. EXPERIMENTAL ENDOTOXIN MASTITIS (IV, V)

7.2.1. Animals

Experimental endotoxin mastitis was induced in 10 Finnish Ayrshire dairy cows. Two cows were primiparous and eight multiparous (2^{nd} - 4^{th} lactation). All cows were clinically healthy, free from mastitis pathogens and had a low milk SCC (<150 000/ml). Cows were housed in a stanchion barn and fed good quality silage and hay freely. Concentrate was given twice daily. They were milked twice a day, at 7:00 and 17:00.

7.2.2. Experimental design

Cows were challenged twice using a cross-over design; in the early lactation (**EL**) period, 6 to 15 days after parturition, and in the late lactation (**LL**) period, 137 to 77 days before the next parturition. The daily milk yield was 17 to 38 kg in EL, and 5 to 19 kg in LL cows. Cows were randomly allocated into two subgroups; one group was challenged first in the EL period and the other group first in the LL period. The endotoxin challenge was done after the morning milking into one hind-quarter with 100 µg of *Escherichia coli* 0111:B4 lipopolysaccharide B (Bacto©, Difco Laboratories, INC., Detroit, USA) diluted in 5 ml of sterile isotonic NaCl. The same quarter was used at both challenges. The contralateral quarter served as a control. During the endotoxin experiment the cows received no treatments. One cow was excluded since it did not conceive. The Ethics Committee of the Faculty of Veterinary Medicine, University of Helsinki, approved the study protocol.

Milk samples were collected from the challenged and control quarters of each cow at 0, 2, 4, 8, 12, 24, 32, 48 and 72 h. Blood samples were collected from the jugular vein into EDTA and serum tubes at the same time-points as the milk samples. Milk samples were stored frozen at -21ºC and serum samples at -70ºC.

7.2.3. Clinical scoring

Systemic and local signs were recorded throughout the experiment. Systemic signs included general attitude, heart rate, rectal temperature and appetite; local signs included swelling, heat, and pain in the udder. Appearance of milk in the challenged quarter was also recorded. Systemic and local signs and milk appearance were scored from 1 to 3, with half numbers also used. In this scoring system, 1 was defined as a normal and 3 as a severe reaction, the latter indicated rectal temperature >40.5˚C, anorexia and depression, severe swelling and pain in the udder and serous or pus-like milk (Pyörälä et al., 1994). The response parameter was formed by calculating the average of these scores. This parameter was handled as a continuous parameter and it fit the normal distribution curve well. Severity of response was assessed as follows: <2 was classified as a mild, ≥2 but <3 as a moderate and 3 as a severe response. Total milk yield was recorded for each cow at both milking times and the 24-h milk yield was calculated.

7.3. METHODS OF ANALYSIS

7.3.1. Virulence factors (I, III)

7.3.1.1. Control strains

Several control strains were used for the detection of different virulence factors by polymerase chain reaction (**PCR**). *E. coli* 25KH9 was used as a positive control for the detection of F17a, *E. coli* S5 for F17b and CNF2, *E. coli* CS31A for F17c and surface antigen 31A, *E. coli* 111KH86 for F17d, *E. coli* BM2-1 for S fimbriae and CNF1, *E. coli* IH87131 for aerobactin and P fimbriae, *E. coli* 262KH89 (provided by Pasteur Institute, France) for Afa7D and Afa7E, *E. coli* 239KH89 (provided by Pasteur Institute, France) for Afa8D and Afa8E, and *Salmonella typhimurium* 2696 for TraT. *E. coli* strain A56 was the positive control for bacteriophage K1, and *E. coli* strain IHE11038 for bacteriophage K5. In the analysis of serum resistance, *E. coli* FT240 was used as a control for sensitive strains, and *E. coli* FT64 for resistant strains (Fang and Pyörälä, 1996).

All strains had been stored either in semi-solid nutrient broth (Lab Lemco Broth, Oxoid, Hampshire, UK) at 20°C or in frozen serum-meat broth with 10% glycerol at -70°C. Strains were refreshed by culturing them on trypticase-soy agar (**TSA**) for 24 h at 37°C.

7.3.1.2. Polymerase chain reaction

PCR was used to analyse the genes of F17-related fimbriae, P fimbriae, S fimbriae, CNF1, CNF2, aerobactin, *E. coli* surface antigen CS31A, afimbrial adhesins of the Afa family and TraT. Analyses were carried out in three multiplex PCRs (**MXP**) (**Table 2**); MXP1 was used to detect the genes for F17a and F17d major subunits and subfamily I adhesin, MXP2 for F17b and F17c major fimbrial subunits and subfamily II adhesin (Bertin et al., 1996), and PAP for P and S fimbriae, CNF1 and aerobactin (Yamamoto et al., 1995). Seven PCR reactions were done individually: clp (Bertin et al., 1998), afa7D, afa7E, afa8D, afa8E (Lalioui et al., 1999), CNF2 and TraT, the last two of which are described in more detail in study **I**.

All PCRs were performed in a total volume of 50 µl, except for the TraT PCR, which was carried out in 25 µl. For the template, 10 µl of fresh bacterial mass from the agar plate was suspended in 500 µl of sterile MilliQ H_2O. One unit of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland) was used for each PCR, except for the 0.5 U used in the clp PCR. Optimized DyNAzyme buffer (Finnzymes, Espoo, Finland) was used as a buffer. Multiplex PCRs MXP1 and MXP2 contained 0.6 µM (30 pmol/50 µl) of each primer, 100 µM of each deoxynucleoside triphosphate (Finnzymes, Espoo, Finland) and 2 µl of the template. The PCRs for CNF2 and Afa were as above, except for the concentration of the primers, which were 0.3 µM and 0.45 µM, respectively. The PAP PCR contained 0.4 µM of each primer, 100 µM of each dNTP, 1.25 mM of Mg^2 and 5 µl of template. The clp PCR contained 0.3 µM of each primer, 100 µM of each dNTP and 3 µl of template.

Six different amplification procedures were used in the UnoII Thermoblock thermal cycler (Biometra, Göttingen, Germany). The parameters were denaturation for 2 min at 95°C and 1 min at 94°C, annealing for 1 min at 55°C for MXP1, TraT and clp, 59°C for MXP2, 52°C for CNF2, 63°C for PAP, 60°C for afa7D, afa8D and afa8E, and 47°C for afa7E, extension for 1 min at 72°C and final extension for 5 min at 72°C. The cycle was repeated 25 times. Nine microliters of the PCR

Table 2. PCR reactions used for the detection of virulence factors.

PCR	Primer	Primer sequence	Product size	Reference strain¤	Virulence factor
MXP1	P2	CTGATAAGCGATGGTGTAATTAAC[1]			
	P3	GCTGGAAGGGTGCAATACGCCTG[1]	321 bp	25KH9	F17a-A
	P6	GATAGTTCATAACCTTAATATTGCA[1]	239 bp	111KH86	F17d-A
	P7	CGGAGCTAATACTGCATCAACC[1]	615 bp		Subfamily I adhesins (F17a-G, F111)
	P9	TGTTGATATTCCGTTAACCGTAC[1]			
MXP2	P2	CTGATAAGCGATGGTGTAATTAAC[1]			
	P4	CAACTAACGGGATGTACAGTTTC[1]	323 bp	S5	F17b-A
	P5	GCAGGAACCGCTCCCTTGGC[1]	416 bp	CS31A	F17c-A
	P8	CGTGGGAAATTATCTATCAACG[1]	615 bp		Subfamily II adhesins (F17b-C, F17c-G)
	P9	TGTTGATATTCCGTTAACCGTAC[1]			
PAP	Pap1	GCAACAGCAACGCTGGTTGCATCAT[4]			
	Pap2	AGAGAGAGCCACTCTTATACGGACA[4]	336 bp	IH87131	P fimbriae
	Sfa1	CTCCGGAGAACTGGGTGCATCTTAC[4]			
	Sfa2	CGGAGGAGTAATTACAAACCTGGCA[4]	410 bp	BM 2-1	S fimbriae
	Aer1	TACCGGATTGTCATATGCAGACCGT[4]			
	Aer2	AATATCTTCCTCCAGTCCGGAGAAG[4]	602 bp	IH87131	Aerobactin
	CNFI	AAGATGGAGTTTCCTATGCAGGAG[4]			
	CNFII	CATTCAGAGTCCTGCCCTCATTATT[4]	498 bp	BM 2-1	CNF1
CNF2	CNF1	ACTGAAGAAGAAGCGTGGAATA[2]			
	CNF2	ATAAGTTGAGCCGAGCGAGG[2]	654 bp	S5	CNF2
TRAT	TraTF	GATGGCTGAACCGTGGTTATG[2]		S. Typhimurium 2696	
	TraTR	CACACGGGTCTGGTATTTATGC[2]	307 bp		TraT
clp	clpG1	GGGCGCTCTCTCCTTCAAC[3]			
	clpG2	CGCCCTAATTGCTGGCGAC[3]	385 bp	31A	CS31A
AFA7D	Afa7D1	GCAGAGCTGAGTCTTGATGTCCGT[6]			
	Afa7D2	CGTAATTATTCCTGTGAACGGCTGTCCA[6]	377 bp	AFA7	Afa7D
AFA7E	Afa7E1	GCTAAATCAACTGTTGATGTT[6]			
	Afa7E2	GGACAATCCAAATGGCGAATTA[6]	618 bp	AFA7	Afa7E
AFA8D	Afa8D1	GTTGAACTGAGTTCTTAATACCAGTG[6]			
	Afa8D2	TGAGCATTCTCCGCTAACTGATAAT[6]	354 bp	AFA8	Afa8D
AFA8E	Afa8E1	CTAACTTGCCATGCTGTGACAGTA[6]			
	Afa8E2	TTATCCCCTGCGTAGTTGTGAATC[6]	302 bp	AFA8	Afa8E

[1] Bertin et al., 1996
[2] This study
[3] Bertin et al., 1998
[4] Yamamoto et al., 1995
[6] Lalioui et al., 1999
¤Strains of *E. coli* if not otherwise stated

product was analysed by agarose gel electrophoresis (140 V, 1 h) in a 1.5% agarose gel containing ethidium bromide. The PCR product was visualized with UV light.

7.3.1.3. Capsule formation

Capsule formation of the strains was examined by using K1 and K5 bacteriophages. Bacteriophages were stored at -70°C as phage lysates. Phage stocks were prepared with the method described by Mäkelä (1985), using *E. coli* A56 (K1) and *E. coli* IHE11038 (K5) as propagating strains.

The isolates and control strains were spread on TSA plates. One drop of 10^{-3} and 10^{-2} diluted K1 and K5 bacteriophage lysates, respectively, was pipetted onto the agar. Plates were air-dried and incubated at 37°C for 24 h. The isolates lysed by capsule-specific phages were regarded as positive for K1 or K5.

7.3.1.4. Serum resistance

Serum resistance was analysed by a turbidometric assay using microtitre plates (Pelkonen and Finne, 1987). Blood for the serum was collected from 14 healthy young cows at a slaughter. Blood was pooled and allowed to clot at room temperature before separation of normal bovine serum (**N-BS**). Inactivated bovine serum (**I-BS**) and bovine serum with inactivated classical complement pathway (**EGTA-BS**) were prepared and stored as described earlier (Pelkonen and Finne, 1987).

The bacterial suspensions were mixed with bovine serum to yield a final serum concentration of 75% and incubated at 37°C for 3 h. The sensitivity to N-BS and EGTA-BS was measured in duplicate. Inactivated serum I-BS was used as a control.

For the turbidimetric assay (Pelkonen and Finne, 1987), 150 µl of serum (N-BS, EGTA-BS or I-BS) was mixed with 50 µl of bacterial suspension. The mixture contained approximately 2 x 10^8 cfu/200 µl. Variation of the concentration between 10^4 and 10^{10} bacterial cells/ml had no effect on serum resistance (Olling, 1977). The first well of the microtitre plate (Titertek, 350 µl, 96 flat-bottom well) served as a blank control, containing 150 µl of N-BS serum and 50 µl of 0.9% NaCl. The absorbance at 620 nm (Labsystems Multiskan MCC/340) was measured at 0, 60, 120 and 180 min. The suspensions were gently mixed with a pipette before every measurement. The initial absorbance values at time zero varied between 0.1 and 0.4. The change in absorbance was calculated as a percentage (remaining absorbance divided by initial absorbance).

7.3.2. Antimicrobial susceptibility testing (II)

7.3.2.1. Control strains

For the detection of minimal inhibition concentration (**MIC**) *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 (NCCLS, 1999) were used as reference strains.
All strains had been stored either in semi-solid nutrient broth (Lab Lemco Broth, Oxoid, Hampshire, UK) at 20°C or in frozen serum-meat broth with 10% glycerol at -70°C. Strains were refreshed by culturing them on TSA for 24 h at 37°C.

7.3.2.2. Antimicrobial agents

MIC values of the isolates were analysed for eight different antimicrobials: ampicillin (ampicillin sodium salt, Sigma Chemical Co., St. Louis, MO, USA, lot 127HO285), cephalexin (cefalexin hydrate, Sigma, lot 24H0420), ceftazidime (ceftazidime pentahydrate, Sigma, lot 35H0473), dihydrostreptomycin (**DHS**) (dihydrostreptomycin sesquisulfate salt, Sigma, lot 66H04142), gentamicin (gentamicin sulfate, Sigma, lot 27H0744), tetracycline (tetracycline, Sigma, lot 38H1324), trimethoprim-sulphadiazine (**TS**) (trimethoprim, Sigma, lot 105H0136; sulfadiatsin, Sigma, lot 96H1175) and ciprofloxacin (ciprofloxacin*HCL, Bayer Ag, Levekusen, Germany, lot 303477A) by an agar dilution method (NCCLS, 1999). The antimicrobial agent stock solutions were prepared and diluted as recommended by their respective manufacturers in a NaCl solution to a concentration of 1280 μg/ml. TS was made up to a ratio of 1:5, so the actual concentrations of these compounds in stock solution were 213.33 μg/ml for trimethoprim and 1066.66 μg/ml for sulphadiazine.

The final concentrations ranged in the two-step dilutions from 128 μg/ml to 0.5 μg/ml for ampicillin, cephalexin, DHS, tetracycline and TS, from 64 μg/ml to 0.25 μg/ml for gentamicin and from 16 μg/ml to 0.0625 μg/ml for ciprofloxacin and ceftazidime, which also had a concentration of 32 μg/ml.

7.3.2.3. Minimal inhibition concentration

The test isolates and reference strains were prepared in a suspension at a concentration of 10^7 cfu/ml (NCCLS, 1999). Antimicrobial agars were inoculated with the respective bacterial solutions using an Automatic Multipoint Inoculator (Mast Diagnostics LTD., Merseyside, UK) according to NCCLS recommendations. Growth of the inoculates was determined after incubation at 37°C for 20 h by observing the agar plates from directly above. The MIC value recorded was the lowest concentration of the antibiotic to inhibit growth of the inoculated bacteria (NCCLS, 1999).

The MIC breakpoint values for *in vitro* susceptibility were 8 μg/ml for ampicillin, cephalexin, DHS and TS, 4 μg/ml for gentamicin and tetracycline (NCCLS, 1999), 0.5 μg/ml for ceftazidime and 0.25 μg/ml for ciprofloxacin. The isolate was considered resistant to a certain antimicrobial when the MIC value was higher than the MIC breakpoint value of that antimicrobial. MIC values for 50% and 90% of isolates tested (**MIC_{90}** and **MIC_{50}**) were calculated for all antimicrobials tested.

7.3.3. Milk inflammation parameters

7.3.3.1. Somatic cell count (IV)

Milk SCC was measured with a Fossomatic instrument (Foss Electric, Hillerød, Denmark); for samples with clots and flakes, a Coulter Counter particle counter (Coulter Electronics Ltd., Northwell, England) was used (IDF, 1991). SCC values were measured with the Coulter Counter at 4, 8 and 24 h, and at other times with the Fossomatic. Values over $15x10^6$/ml were recorded as $15x10^6$/ml.

7.3.3.2. N-acetyl-β-D-glucosaminidase (III, IV)

Milk NAGase activity was measured using a commercial Milk NAGase kit (Applied Diagnostics Corporation, Helsinki, Finland) (Mattila and Sandholm, 1986).

7.3.4. Haematological parameters (IV, V)

Haematological parameters (packed cell volume (**PCV**), haemoglobin, total and differential leucocyte count) were determined within 2 h of sampling using an automated multiparameter analyser with software for animal samples (CELL-DYN 3700 System, ABBOTT Diagnostic Division, ABBOTT Park, IL, USA).

7.3.5. Serum biochemical parameters (IV)

Serum cortisol levels were measured by radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Product Corporation, Helsinki, Finland). Serum urea and creatinine were measured with an enzymatic kinetic method (Fabiny and Ertigshausen, 1971; Gutmann and Bergmeyer, 1974) using an automated analyser (KONE Pro, ThermoClinical Labsystems, Espoo, Finland). Another automated analyser (KONE Specific, ThermoClinical Labsystems, Espoo, Finland) was used to determine serum aspartate aminotransferase (**ASAT**) activity (Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974) and free fatty acids (**FFA**) concentration (Waco Chemicals GmbH, Neuss, Germany). The KONE Lab-Microlyte 3+2 (ThermoClinical Labsystems, Espoo, Finland) analyser was used to determine serum Ca^{2+}.

7.3.6. Neutrophil function (IV)

PMN phagocytosis and respiratory burst in blood and milk were determined by zymosan-induced CL as described earlier (Lilius and Waris, 1984; Lojek et al., 1997). Briefly, 20 µl of luminol (10 mM), 25 µl of opsonized zymosan (20 mg/ml), 180 µl of HBSS (0.1% gelatin) and 25 µl of EDTA blood (1:200 dilution) or milk (1:1000 dilution) were pipetted into the wells of a microtitre plate, and the CL was measured at 37°C for 40 min with a luminometer (Luminoskan, Labsystems, Helsinki, Finland). The samples were analysed in triplicate within 2 h of sampling. The highest CL value during the 40-min measuring time was considered to be the CL value of that time-point. The value was divided by the number of PMN in blood or the number of somatic cells in milk at that time to obtain the CL of one cell.

7.3.7. Tumour necrosis factor alpha (V)

The enzyme-linked immunosorbent assay (**ELISA**) used in this study was modified from Ellis et al. (1993) by Røntved et al. (unpublished results).

The following adjustments were made for the quantification of TNFα in serum. Serum samples were diluted 1:2 in tris-buffered saline (**TBS**: 0.05 M Tris, 0.15 M NaCl, pH 7.6) containing 0.05% tween and 0.5% gelatin (Sigma-Aldrich Co., St. Louis, USA) (TBS-T-g), and duplicates of the samples were added to two coated plates and incubated overnight at 4°C. A 2-fold dilution of recombinant bovine TNFα (Ciba-Geigy, Basel, Switzerland) in 50% TBS-T-g and 50% FCS was used as the standard, starting at 62.5 ng/ml. A high and a low positive control were added in a ratio of 1:2. A mixture of 50% TBS-T-g and 50% FCS was used as a negative control.

The same ELISA was slightly modified to analyse TNFα in milk samples. Before TNFα analysis, milk samples were centrifuged at 25.000 g for 40 min at 4°C. Fat was removed and the supernatant collected; the supernatant was then centrifuged again at 2000 g for 20 min at 4°C to remove any remaining fat. The positive and negative controls for milk samples were treated in a similar way. The milk supernatants were diluted from 1:4 to 1:512 (2-fold dilution) in TBS-T-g to find the most appropriate dilution. The diluted samples were tested in triplicate. The samples were then added to coated plates and incubated overnight at 4°C. A 2-fold dilution of recombinant bovine TNFα in TBS-T-g with 6.25% milk supernatant was used as a standard, starting at 62.5 ng/ml (the bo rTNFα stock was 3.125 µg/ml and kept in TBT-g with 10% heparin stabilized bovine plasma). A high positive control was added in dilutions of 1:64 and 1:128, and a low positive control in a dilution of 1:8; TBS-T-g with 6.25% milk supernatant was used as a negative control. The following day, the procedure was continued as described above.

The interassay (between days) and intraplate coefficients of variation for the serum TNFα ELISA were below 14.4% and 14.8%, respectively, and for the milk TNFα ELISA as follows: for the low control (5.8 ng/ml diluted 1:8) 9.8% and 6.7%, and for the high control (59.5 ng/ml diluted 1:64) 6.6% and 9.1%, respectively. The detection limit of the ELISA was 0.5 ng/ml for the serum and around 1.0 ng/ml for the milk.

7.3.8. Serum amyloid A (V)

The concentration of SAA in serum and milk was determined by using a commercial ELISA kit (Tridelta Development, Wicklow, Ireland) and as described by Eckersall et al. (2001). For high SAA concentrations, the samples were diluted if necessary. The detection level of SAA was >0.3 µg/ml for both serum and milk samples, and the upper limit for the analysis was <750 µg/ml. The inter-assay and intra-assay coefficients of variation for SAA analysis were <10 % and < 5 %.

7.4. STATISTICS

Pearson's chi-square test was used to study differences in the frequencies of resistance to the antimicrobials tested among the isolates (**II**) and in the association of F17-related fimbriae, P and S fimbriae (grouped together), CNF1 and CNF2, aerobactin, TraT, serum resistance and the clinical course of mastitis with the lactation stage (**III**). Multiple comparisons were carried out between different factors and their combinations.

Fisher's exact test was used to examine the association of F17-related fimbriae, P and S fimbriae (grouped together), CNF1 and CNF2, aerobactin, TraT and serum resistance with the clinical course of mastitis (**III**).

Analysis of variance was used to study the association of F17-related fimbriae, P and S fimbriae (grouped together), CNF1 and CNF2, aerobactin, TraT, serum resistance and the clinical course of mastitis with milk NAGase activity (**III**).

Repeated measures analysis of variance was used to test the effect of lactation stage on systemic signs, local signs, milk appearance, rectal temperature, TNFα in milk and SAA in serum and in milk. Significance of hour and stage*hour effect were evaluated by Greenhouse-Geisser adjusted *P* values (**V**).

A mixed linear model (Lindsay, 1993) with two different models were used to test the effect of lactation stage on logNAGase, logSCC, WBC, leucocyte count, neutrophil count, PCV, haemoglobin, CL in milk and in serum, urea, creatinine, cortisol, FFA, ASAT and Ca^{2+}. All estimates were calculated using the MIXED procedure in SAS statistical software (SAS 2002, SAS Institute Inc., Gary, NC, USA) (**IV**).

In all statistical analyses, a *P* value of <0.05 was considered significant.

8. RESULTS

8.1. FIELD DATA

8.1.1. Characteristics of mastitis (III)

Of the 155 Finnish clinical mastitis cases caused by *E. coli*, 77 were categorized as mild and 78 as severe. Sixteen cows had persistent *E. coli* mastitis, 116 had non-persistent mastitis and 26 missed the resampling. Severity of the acute stage in persistent and non-persistent mastitis did not differ significantly. Milk NAGase activity was significantly ($P<0.01$) lower in severe than in mild mastitis (221 $\pm$ 23 arbitrary units (**AU**) vs. 291 $\pm$ 21 AU). Thirty-two per cent of clinical cases of mastitis occurred in EL (0-21 days PP), 54% in mid-lactation (21-210 days PP) and 14% in LL (over 211 days PP). Lactation stage was significantly ($P<0.05$) associated with severity: clinical signs tended to be milder EL than in LL (**Figure 2**). Lactation stage did not affect the persistence of mastitis or the presence of any virulence factors.

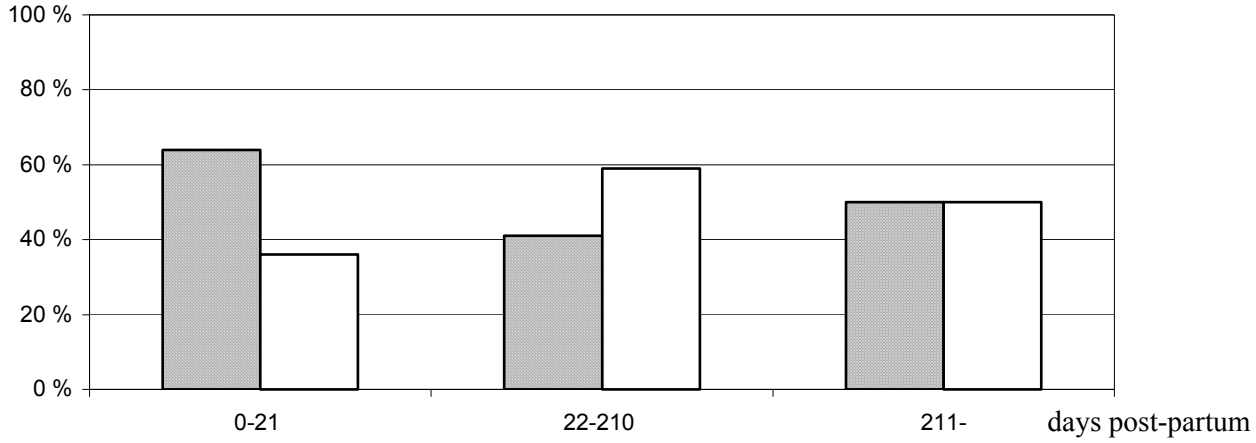


Figure 2. Distribution of cows with mild (dashed bar) and severe (open bar) course of *E. coli* mastitis according to lactation stage. The number of cows in early lactation (0-21 days post-partum) was 50, in mid-lactation (22-210 days post-partum) 83 and in late lactation (over 211 days post-partum) 22.

8.1.2. Virulence factors of *E. coli* isolates (I)

Of the 160 Finnish and 113 Israeli *E. coli* isolates, 79 (49%) and 48 (42%), respectively, had at least one virulence gene detected by PCR. The most common gene in both Finnish and Israeli isolates was *traT* (**Table 3**). It was detected alone in 52% of Finnish and 88% of Israeli isolates positive for any virulence gene. Other genes were rare in Israeli isolates (**Table 3**). None of the isolates contained genes for F17a, Afa7D, Afa7E and CS31A .

Most of the virulence genes were detected in different combinations. The combinations varied markedly and each combination was typically present in only a few isolates. Two major groups of combinations were detected; those with F17-related genes and those with genes for S and P fimbriae and CNF1 (**Table 4**). The genes for CNF2, aerobactin and TraT were found in both groups.

Table 3. Prevalence of virulence factors among *E. coli* isolated from mastitis.

Virulence factor	Finnish isolates (n=160)		Israeli isolates (n= 113)	
	n	%	n	%
F17a	0	0	0	0
F17b	1	0.6	1	0.9
F17II	5	3.1	0	0
F17c	4	2.5	0	0
F17d	5	3.1	0	0
S fimbria	12	7.5	1	0.9
P fimbria	11	6.9	0	0
CS31A	0	0	0	0
Afa7D	0	0	0	0
Afa7E	0	0	0	0
Afa8D	2	1.3	0	0
Afa8E	2	1.3	0	0
CNF2	23	14.4	4	3.5
CNF1	12	7.5	1	0.9
aerobactin	18	11.3	5	4.4
TraT	59	36.9	46	40.7
Negative	81	51.0	65	58.0

Table 4. Different combinations of virulence genes detected among Finnish and Israeli *E. coli* isolates.

Combinations of genes	Finnish isolates	Israeli isolates
F17	3	0
F17, traT	1	0
F17, afa8DE	1	0
F17, cnf2, aer	1	0
F17, cnf2, traT	2	0
F17, cnf2, aer, traT	4	1
F17, cnf2, afa8DE, aer	1	0
F17, cnf2	1	0
SP, cnf2, cnf1, aer, traT	1	1
SP, cnf2, cnf1, aer	1	0
SP, cnf2, cnf1	6	0
SP, cnf1	4	0
cnf2, aer, traT	4	1
cnf2	2	1
aer, traT	6	2
traT	41	42
Negative	81	65
Total number	160	113

E. coli isolates were divided into the following four groups according to their growth in 75% bovine serum: resistant, intermediate, slow-intermediate and sensitive. Resistant isolates continued growth throughout the incubation period. Intermediate isolates also continued growing but more slowly than resistant strains. Slow-intermediate isolates grew rapidly for the first 60 min but then showed lysis. Sensitive isolates grew only weakly and after 60 min showed clear lysis. Only 6% of the Finnish isolates were defined as serum-sensitive, 7% were slow-intermediate, 29% were intermediate and 59% were clearly resistant to serum. Among the Israeli isolates, 31% were sensitive, 15% slow-intermediate, 30% intermediate and 17% clearly resistant. The Finnish strains overall were more resistant to serum than the Israeli strains. Strains classified as resistant or intermediate had more genes for virulence factors than strains classified as sensitive, slow-intermediate or sensitive to I-BS. This trend was the same in both Finnish and Israeli strains (**Table 4**). For all groups, similar growth curves were observed in N-BS and EGTA-BS. Eight Israeli isolates were sensitive to I-BS and could not be analysed further.

All isolates lacked K1 and K5 capsules.

8.1.3. Antimicrobial resistance (II)

The MIC values of *E. coli* isolates from both Finland and Israel were quite similar and relatively low (**Table 5**). The majority of the isolates were susceptible to the antimicrobial agents studied. A total of 30% of Finnish and 24% of Israeli isolates were resistant to one or more antimicrobials. No gentamicin-, ciprofloxacin- or ceftazidime-resistant isolates were detected. The only significant ($P<0.01$) difference in the number of resistant isolates between the two countries was for cephalexin; 16% of Finnish isolates but only 3% of Israeli isolates were resistant to this first-generation cephalosporin. For the other antimicrobial agents, the proportions of resistant strains were higher for Israeli isolates than for Finnish ones, although the differences were not significant (**Table 5**).

While MIC_{50} values for Finnish and Israeli isolates did not differ markedly, differences were seen between MIC_{90} values (**Table 5**). For cephalexin, the Finnish isolates had one dilution higher MIC_{90} values than Israeli isolates, whereas for ceftazidime and TS the MIC_{90} values were one dilution higher among Israeli than among Finnish isolates. For DHS and tetracycline, Israeli isolates had clearly higher MIC_{90} values than Finnish isolates.

Thirty-two isolates, 20 from Finland and 12 from Israel, were resistant to only one antimicrobial, whereas 22 isolates, 10 Finnish and 12 Israeli, were resistant to two or more antibiotics (**Table 6**). Of the total amount of resistant isolates, 41% were multiresistant. They formed 10 different combinations; tetracycline was included in all except one combination (**Table 6**).

Table 5. Number of resistant isolates, MIC_{50}[1] and MIC_{90}[2] values of the tested *E. coli* isolates from Finland and Israel (n=100 isolates/country).

Antimicrobials	MIC[3] break-point values (μg/ml)	Range of the MIC values (μg/ml)	No of resistant isolates		MIC_{50} (μg/ml)		MIC_{90} (μg/ml)	
			Finnish	Israeli	Finnish	Israeli	Finnish	Israeli
Ampicillin	8[4]	1- >128	7	10	4	4	8	8
Cephalexin	8[4]	2- >128	16*	3	8	8	16	8
Ceftazidime	0.5	<0.0625- 1	0	0	0.125	0.25	0.25	0.5
DHS[5]	8[4]	<0.5- >128	9	13	4	2	8	32
Gentamicin	4[4]	<0.25- 4	0	0	1	0.5	1	1
Tetracycline	4[4]	1- >128	14	15	4	2	8	64
TS[6]	8[4]	<0.5- >128	2	4	0.25	2	2	4
Ciprofloxacin	0.25	<0.0625- 0.125	0	0	<0.0625	<0.0625	<0.0625	<0.0625

[1] Minimal inhibition concentration for 50% of isolates tested
[2] Minimal inhibition concentration for 90% of isolates tested
[3] Minimal inhibition concentration
[4] According to the NCCLS (1999)
[5] Dihydrostreptomycin
[6] Trimethoprim-sulfadiazine
* Significant ($p<0.01$) difference between countries

Table 6. Resistance patterns among *E. coli* strains isolated from bovine mastitis in Finland and Israel.

Resistance pattern					Number of isolates	
					Finnish	Israeli
Tetracycline	DHS[1]	Ampicillin	Cephalexin	TS[2]	1	0
Tetracycline	DHS	Ampicillin	Cephalexin		1	0
Tetracycline	DHS	Ampicillin	TS		0	4
Tetracycline	DHS	Ampicillin			0	1
Tetracycline	DHS	Cephalexin			1	0
Tetracycline	DHS	TS			1	0
Tetracycline	DHS				1	4
Tetracycline	Ampicillin	Cephalexin			1	0
Tetracycline	Ampicillin				2	1
DHS	Ampicillin				2	2
Tetracycline					6	5
DHS					2	2
Ampicillin					0	2
Cephalexin					12	3
Negative					70	76
Total					100	100

[1] dihydrostreptomycin
[2] trimethoprim-sulfadiazine

8.1.4. Association of virulence factors and antimicrobial resistance with clinical characteristics and outcome of mastitis (III)

Neither virulence factors nor antimicrobial resistance was significantly associated with the severity of mastitis (**Figure 3**). Isolates positive for S and P fimbriae ($P<0.001$), CNF1 ($P<0.001$) and CNF2 ($P<0.05$) were significantly associated with persistent mastitis (**Figure 4**). NAGase activity of milk in cases with *E. coli* isolates positive for S and P fimbriae and CNF1 was on average 389 ± 47.6 AU, which was significantly ($P<0.01$) higher than with isolates negative for these virulence factors, average activity being 245 ± 14.5 AU. In F17-positive isolates, NAGase activity was significantly ($P<0.01$) lower as compared with the cases caused by bacteria without these virulence factors (146 ± 33.8 AU and 268 ± 17.0 AU).

Some virulence factors were associated with certain antimicrobial resistance patterns. A significant association were present between tetracycline resistance and possession of virulence gene *cnf1*, *cnf2*, *SP*, *aer* ($P<0.01$) or *traT* ($P<0.05$). These virulence genes were often found in the same isolates (**Table 4**). A significant ($P<0.05$) association was also observed between DHS resistance and possession of *F17*, *cnf2* or *aer* virulence genes, and between ampicillin resistance and the gene for aerobactin.

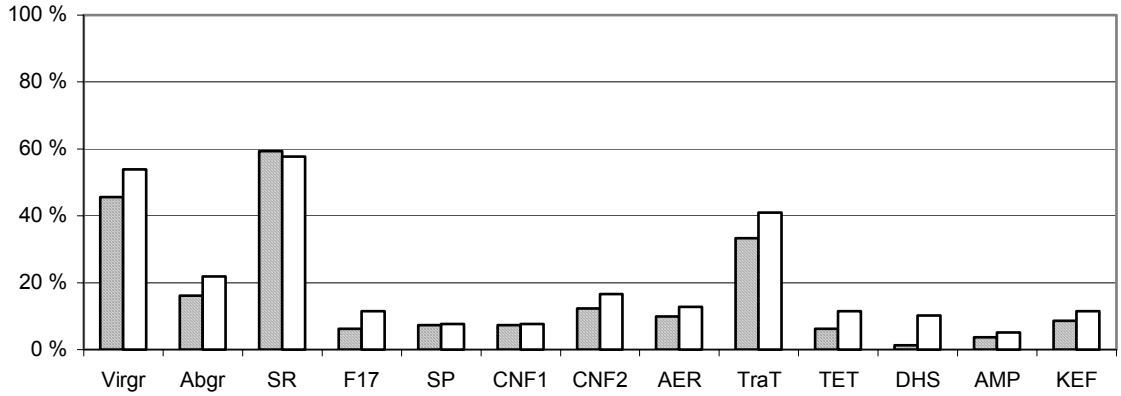
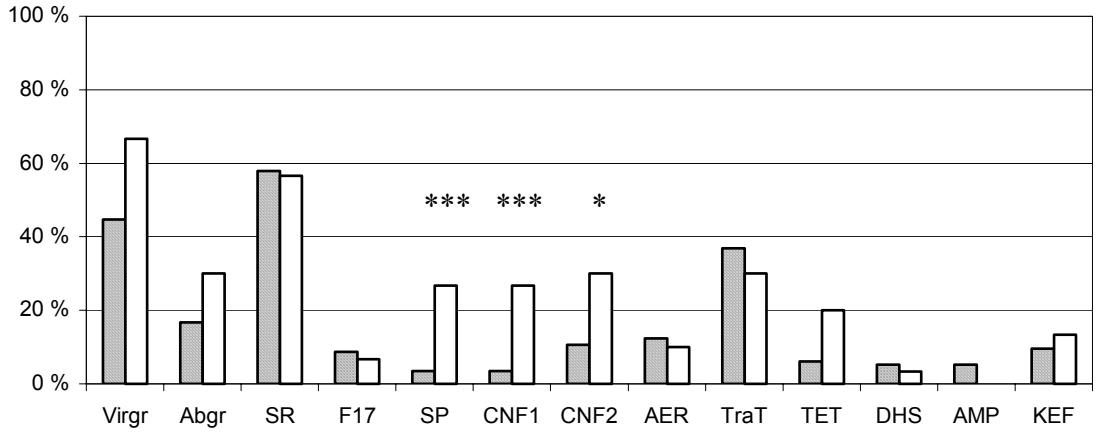


Figure 3. Proportion of *E. coli* isolates (n=155) causing mild (dashed bar) or severe (open bar) course of mastitis in relation to certain virulence factors or antimicrobial resistance; Virgr= positive for any virulence factor, Abgr=resistant to any antimicrobial agent, SR= serum resistant, F17= positive for F17 fimbriae, SP= positive to S or P fimbriae, CNF1= positive for CNF1, CNF2= positive for CNF2, AER= positive for aerobactin, TraT= positive for TraT, TET= resistant to tetracycline, DHS= resistant to DHS, AMP=resistant to ampicillin, KEF= resistant to cephalexin.



*P<0.05
***P<0.001

Figure 4. Proportion of *E. coli* isolates (n=155) causing non-persistent (dashed bar) or persistent (open bar) course of mastitis in relation to certain virulence factors or antimicrobial resistance. A significant difference is indicated with an asterisk. Legends as in Figure 3.

8.2. EXPERIMENTAL ENDOTOXIN MASTITIS

8.2.1. Mastitis outcome (IV)

In experimental endotoxin mastitis, all cows developed clinical signs at both challenges, although none of the cows had a severe response. Clinical response, including systemic and local signs and milk appearance, was more severe in EL than in LL. Systemic signs peaked between 4 and 8 h PC and returned to normal by 32 h PC (Figure 5). Rectal temperatures started to increase at 2 h PC, and this increase was significantly (P<0.01) more pronounced in EL than in LL between 2 and 4 h PC. The peak temperature occurred at 8 h PC, being on average 40.3˚C in EL and 40.1˚C in LL. Severe or moderate local signs were present in the udder already at 2 h PC, peaking at 4 h PC

(**Figure 5**). Changes in milk appearance followed local signs in the quarters (**Figure 5**): milk turned serous and yellow on average at 8 h PC in EL and at 4 h PC in LL. More severe changes in milk appearance were seen in LL than in EL, but individual variation was substantial. The severity of systemic signs varied more than that of local signs between cows and lactation stages.

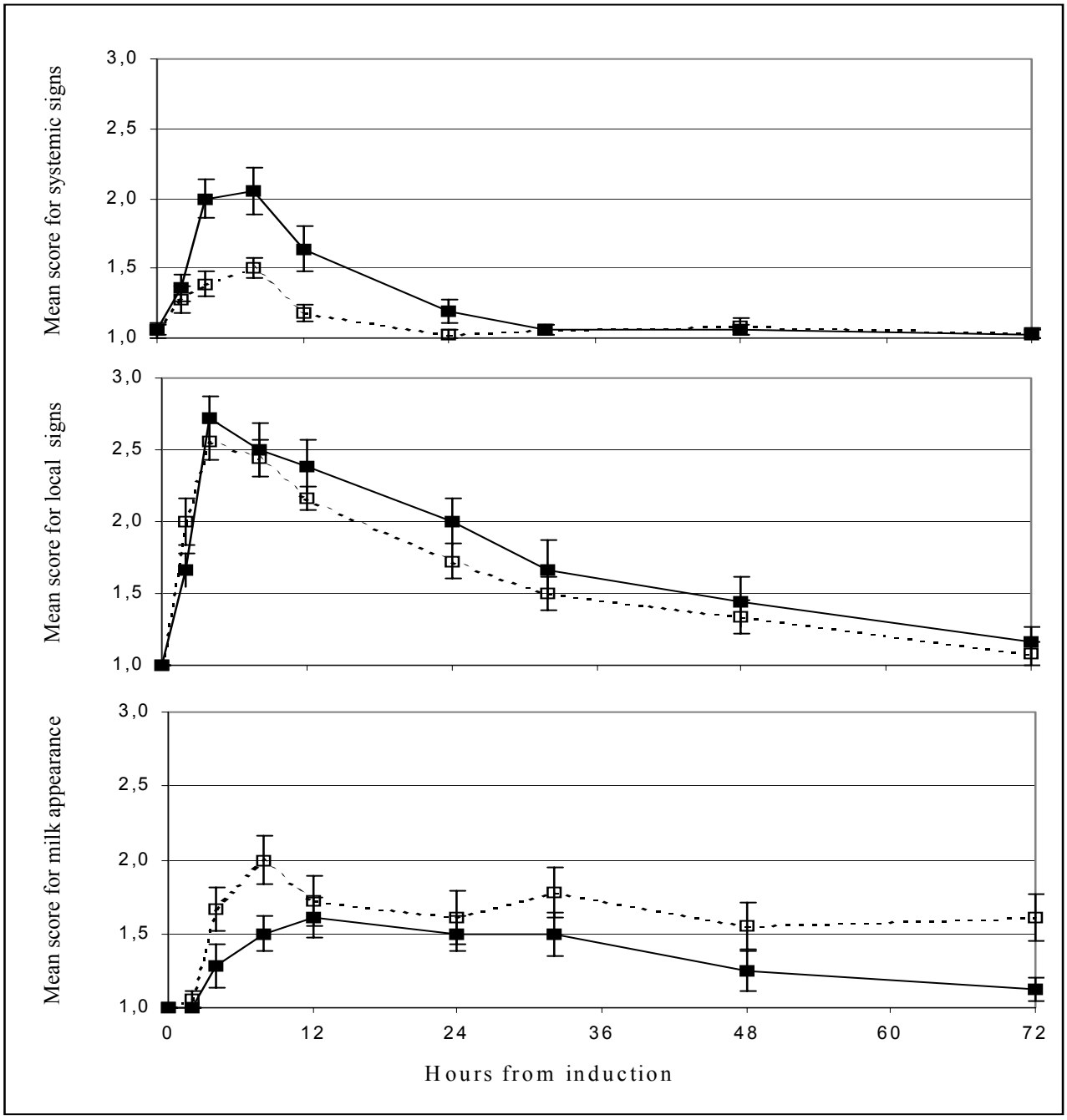


Figure 5. Change in systemic and local signs and milk appearance of cows after induction of endotoxin mastitis. Data points represent the average (±SE) of nine cows. Values in early lactation are indicated with solid symbols and lines, and those in late lactation with open symbols and dashed lines.

Only mild changes in milk appearance occurred in the control quarters. Daily milk yield decreased in most cows on the first day after challenge, on average 18% in EL and 35% in LL. The milk yield returned to baseline levels on the second day in EL and on the third day in LL.

8.2.2. Serum and milk parameters (IV)

Prechallenge values for serum biochemistry and milk inflammatory parameters were generally similar between cows in EL and LL. Significant differences were found only for milk yield, serum urea and FFA concentration (**Table 7**).

Table 7. Initial values of parameters studied in nine dairy cows in early and late lactation. A significant difference between columns is indicated with an asterisk.

	Early lactation		Late lactation	
Parameter	Average	SD	Average	SD
Temperature (ºC)	38.6	0.20	38.7	0.37
Heart rate (/min)	83	13.6	82	11.2
Milk yield (kg/24 h)	27.7***	6.2	13.8	4.6
SCC (x10^3/ml)	37	29.4	83	163.9
NAGase (AU^3)	22	6.7	15	15.7
WBC (x10^6/ml)	7.98	1.49	7.83	1.67
Neutrophils (x10^6/ml)	3.53	1.33	3.05	1.01
Lymphocytes (x10^6/ml)	3.06	0.58	3.48	0.81
Haematocrite (%)	34.7	1.6	32.6	2.9
Ca^{2+} (mmol/l)	1.31	0.13	1.28	0.09
ASAT (u/l)	89	16.7	73	20.2
Urea (mmol/l)	3.6*	0.71	4.6	0.61
Creatine (µmol/l)	90	9.7	88	5.2
FFA (mEq/l)	0.37***	0.14	0.16	0.04
Cortisol (nmol/l)	11.36	8.82	10.90	10.17
CL in milk (RLU/1000 cells)	0	0	0	0
CL in blood (RLU/1000 cells)	0.12	0.06	0.13	0.04
TNFα in milk (µg/ml)	0	0	0	0
TNFα in serum (µg/ml)	0	0	0	0
SAA in milk (µg/ml)	0.34	0.5	0.3	0.6
SAA in serum (µg/ml)	13.01	13.63	16.77	20.49

SCC = somatic cell count
NAGase = N-acetyl-β-D-glucosaminidase
AU = Arbitrary units
WBC = White blood cell count
ASAT = Serum aspartate aminotransferase activity
FFA = Free fatty acids
CL = chemiluminescence
RLU = relative light unit
TNFα = Tumour necrosis factor alpha
SAA = Serum amyloid A
*$P<0.05$
***$P<0.001$

In the endotoxin-infused quarter, milk SCC clearly started to increase at 2 h PC, reaching a maximum at 8-24 h PC (**Figure 6**). Milk NAGase activity followed the increase of SCC, peaking at 12 h PC at 320 AU in EL and 269 AU in LL cows. EL cows had a significantly (P<0.05) higher SCC than LL cows. NAGase levels were also higher in EL, but the difference was not significant. SCC and NAGase did not return to baseline levels during the experimental period. Milk SCC and NAGase from the control quarters stayed within the normal range in both EL and LL.

The white blood cell (**WBC**) count changed significantly (P<0.001) during the experiment. At 4 h PC, the WBC count was its lowest, at 5.3 x 10^6 cells/ml in EL and 5.2 x 10^6 cells/ml in LL. The peak WBC count, 13.3 x 10^6 cells/ml and 9.4 x 10^6 cells/ml, was seen at 24 h PC in EL and at 32 h PC in LL, respectively. The difference in the average levels of WBC between EL and LL was significant (P<0.01), being higher in EL. The number of blood neutrophils also changed significantly (P<0.001) during the experiment for both EL and LL, and was significantly (P<0.01) different between EL and LL cows (**Figure 7**). After 4 h PC, the increase in the neutrophil count was more pronounced in EL than in LL, remaining higher until 32 h PC. In addition, the number of blood lymphocytes changed markedly (P<0.001) during the experiment for both lactation stages (**Figure 7**). At 8 and 12 h PC, the number of lymphocytes was lower in EL than in LL. While the PCV changed significantly (P<0.001) during the experiment, the values stayed within normal ranges.

Serum cortisol changed significantly (P<0.001) during the experiment, reaching peak values at 4 h PC (mean values 134.8 nmol/l in EL and 156.4 nmol/l in LL) and returning to baseline levels after 12 h PC. No significant differences were seen between the levels in EL and LL. Concentrations of serum urea, FFA, creatinine and ASAT also showed significant (P<0.001) changes during the experiment, but the values stayed within the normal range. The concentrations of serum FFA were markedly (P<0.001) higher and serum urea significantly (P<0.05) lower in EL than in LL throughout the study. The changes in blood Ca^{2+} levels were not significant.

8.2.2.1. Neutrophil function (IV)

After the endotoxin challenge, the CL of blood neutrophils started to decrease in EL and increase in LL (**Figure 7**), whereas in the milk CL increased in both EL and LL (**Figure 6**). The increase was more prominent in EL, although the differences were not statistically significant. CL changes during the experiment were not significantly different from baseline for either blood or milk.

8.2.2.2. Tumour necrosis factor alpha (V)

No detectable levels of TNFα in serum were found at any time-point. The concentration of TNFα in milk began to increase after the endotoxin infusion, peaking at 8 h PC (**Figure 6**). The peak concentration was higher in EL than in LL, at an average of 111.9 ng/ml vs. 33.9 ng/ml, although the difference was not significant at any time-point between lactation stages.

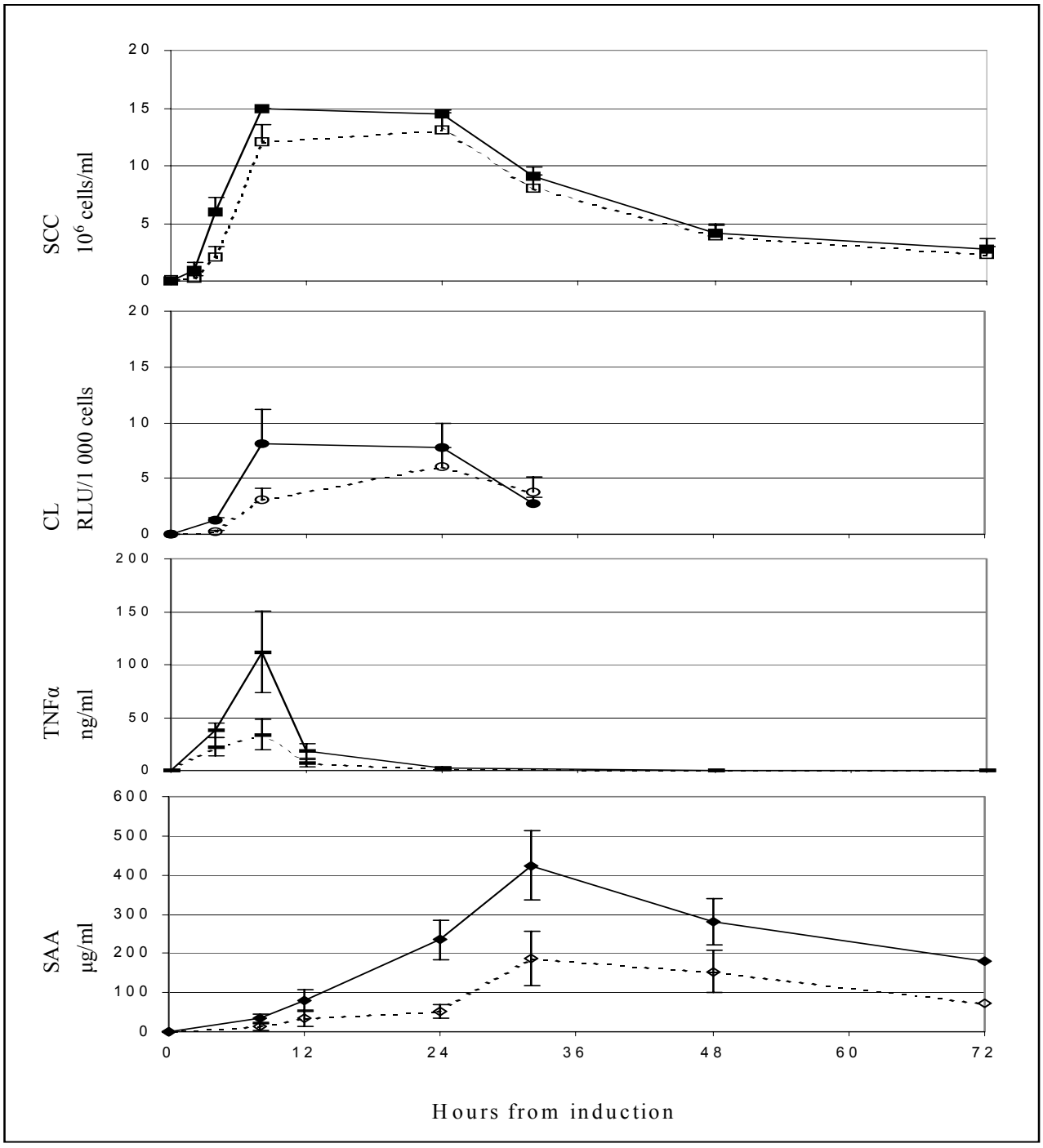


Figure 6. Changes in milk somatic cell count, chemiluminescence and concentrations of TNFα and serum amyloid A in milk after induction of endotoxin mastitis. Data points represent the average (±SE) of nine cows. Values in early lactation are indicated with solid symbols and solid lines, and those in late lactation with open symbols and dashed lines.

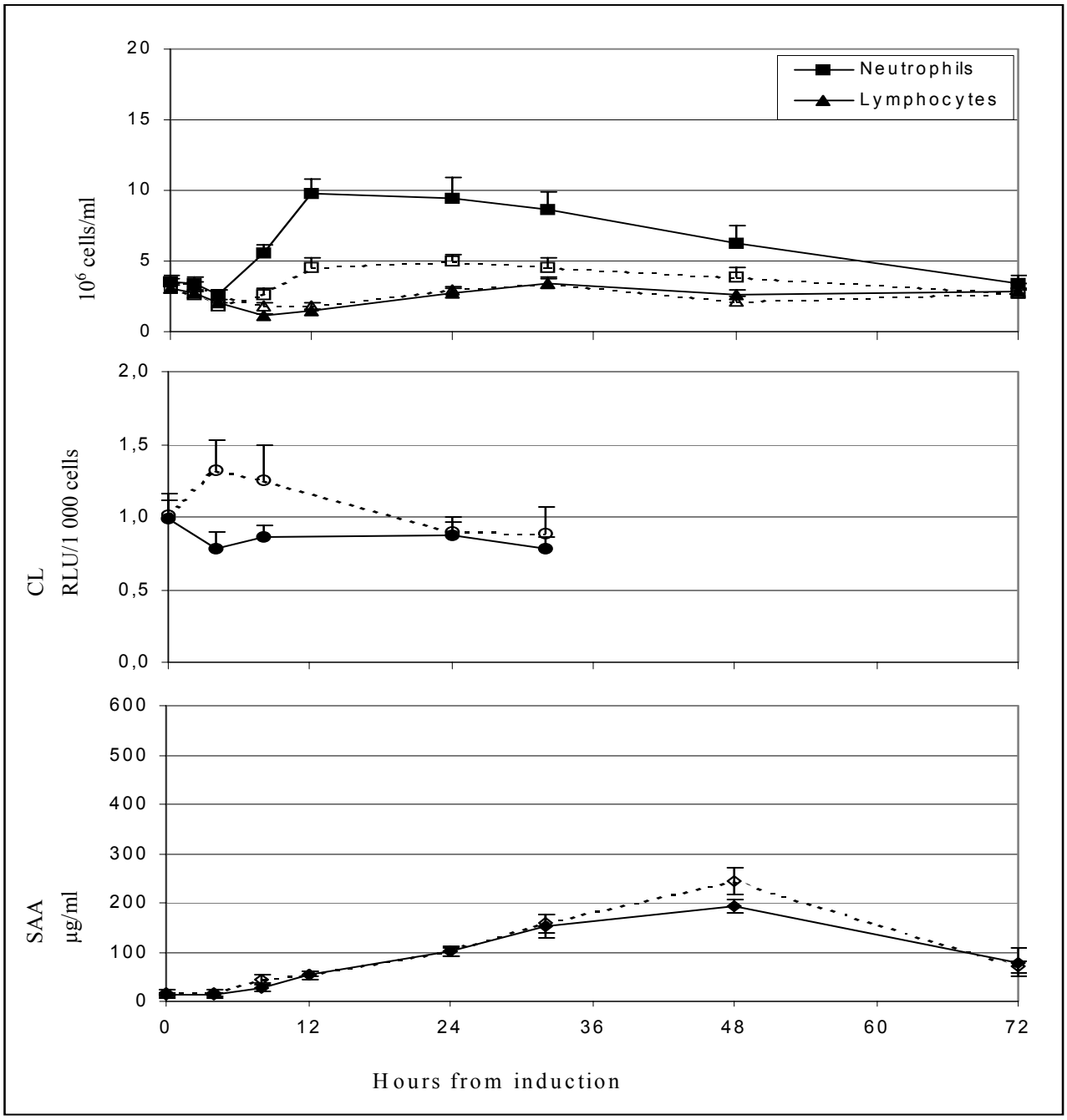


Figure 7. Changes in blood neutrophils and lymphocytes, chemiluminescence, and concentration of serum amyloid A in serum after induction of endotoxin mastitis. Data points represent the average (±SE) of nine cows. Values in early lactation are indicated with solid symbols and solid lines, and those in late lactation with open symbols and dashed lines.

8.2.2.3. Serum amyloid A (V)

Intramammary endotoxin caused a highly significant (P<0.001) increase in both serum and milk SAA concentrations. Initial values were on average 14.9 (range 2.1-67.1) μg/ml in serum and close to the lower detection limit (0.3 μg/ml) in milk; no significant differences between EL and LL were seen. The concentrations started to increase after 8 h PC, peaking at 32 h PC in milk (**Figure 6**) and at 48 h PC in serum (**Figure 7**). Compared with prechallenge values, SAA concentration in serum was significantly higher after 24 h PC in EL (P<0.001) and after 12 h PC in LL (P<0.05). In milk, significantly higher values were seen already after 8 h PC in EL (P<0.05) and after 24 h PC in LL (P<0.05).

The individual time-concentration curves of SAA in serum were similar in EL and LL. The average peak concentration at 48 h PC was significantly (P<0.05) higher in LL than in EL (**Figure 7**), at 244.3 μg/ml and 193.5 μg/ml, respectively.

In milk, SAA concentrations were higher in EL than in LL (**Figure 6**). Between 12 and 24 h PC, the SAA concentrations increased significantly (P<0.05) more in EL than in LL, and statistically different values were seen at 24 h (P<0.01) and 32 h PC (P<0.05). The average peak concentrations were 424.6 μg/ml in EL and 185.8 μg/ml in LL at 32 h PC.

9. DISCUSSION

Pathogenesis of bacterial infection involves a complicated interaction between bacterial and host factors. In most *E. coli* infections, the pathogenicity of the bacterial strain is obligatory to this interaction and defines the course of the disease. Mastitis caused by *E. coli* seems to differ from other *E. coli* infections in its pathogenesis. A normal healthy udder is free from all pathogens. Invasion of infective agents activates a host response and clinical signs appear. *E. coli* mastitis differs from other *E. coli* infections because the clinical signs are primarily caused by the host response and not by the pathological changes derived from bacterial toxins and other damaging factors.

Several previous studies have been published about the initiation, severity and outcome of *E. coli* mastitis, and the possible effects of different bacterial and host factors. Strong evidence shows that *E. coli* mastitis is not caused by a limited number of strains with certain specific characteristics but by a great variety of strains arising from the environment of the cow (Linton et al., 1979; Linton and Robinson, 1984; Sanchez-Carlo et al., 1984b; Valente et al., 1988; Lipman et al., 1995; Jung, 1999). Moreover, the cow's host response has been demonstrated to be a key factor in determining susceptibility to *E. coli* mastitis (Burvenich et al., 2003). Our study confirms earlier results which have indicated that *E. coli* isolates from clinical bovine mastitis possess a variety of different virulence factors but that these factors are not common. None of the virulence factors found here was associated with disease severity.

Among all 273 *E. coli* isolates studied, 66% had a gene for some virulence factor or were resistant to serum. Different virulence factors were distributed widely, and 29 different combinations of virulence factors were detected, including different groups of serum resistance. This is in agreement with earlier results which have suggested that no special virulence characteristics are commonly present among *E. coli* strains isolated from mastitis (**Table 1**). To infect the bovine udder, specific virulence factors, e.g. those enhancing adhesion or invasion in the epithelia or damaging host cells, seem not to be necessary for *E. coli* bacteria. For the pathogenesis of *E. coli* mastitis, the only essential event appears to be the release of endotoxin, which initiates a host response in mastitis but has no direct toxic effect (Cullor, 1996; Hogan and Smith, 2003).

Adhesion and invasion of bacteria to the host epithelia is considered the first event in bacterial infections. Different fimbriae and adhesins are crucial to the pathogenicity of *E. coli* strains, causing diarrhoea and urinary infections. In cattle populations, *E. coli* with F17 fimbriae are very common. In Finland, 49% of healthy calves or calves with diarrhoea carry *E. coli* strains positive for fimbrial adhesins, such as F17 fimbriae, in the intestine (T. Pohjanvirta, personal communication). Afimbrial adhesins are also found from *E. coli* isolated from intestinal and extraintestinal sources of cattle (Bouguénec and Bertin, 1999). For example, S and P fimbriae are important in adhesion of uropathogenic *E. coli* strains (Soto and Hultgren, 1999).

In *E. coli* isolated from mastitis, only Lipman et al. (1995) and Stephan and Kuhn (1999) have shown the presence of genes coding fimbriae production (**Table 1**). Adhesion or attachment of bacteria to the mammary epithelium is not considered important in the pathogenesis of the disease (Opdebeek et al., 1988). Our findings support this since less than 10% of all isolates were positive to any fimbriae or adhesins studied. Prevalence of the genes for F17-related fimbriae was only 9% in the Finnish strains and 1% in the Israeli strains. The S and P fimbriae were generally found together in the Finnish isolates, 7.5% being positive for S fimbriae and 7% for P fimbriae. Of the Israeli isolates, only one was positive for S fimbriae, and none of the isolates had P fimbriae. Two

groups of afimbrial adhesins were also tested, Afa and CS31A. These were rare in the isolates studied here; genes for Afa7 or CS31A were not found at all, and only two Finnish isolates possessed the gene for Afa8.

Toxin production is another common virulence factor of *E. coli*. After colonization, some strains produce toxins that have a deleterious effect on host cells, disturbing their functions. Production of CNF2 is common among bovine *E. coli* strains. The prevalence of CNF-positive *E. coli* strains among isolates from healthy cattle is 30-45% (Pohl et al., 1993; Burns et al., 1996), and from healthy calves and calves with diarrhoea, 71% and 61%, respectively (T. Pohjanvirta, a personal communication). Only one CNF2-producing *E. coli* strain has previously been reported in relation to mastitis (Pohl et al., 1993), indicating that toxin production may not be involved in the disease pathogenesis. However, the number of *E. coli* isolates used to determine CNF2 production has been low, in total only 91 isolates **(Table 1)**. In our material, CNF2 was the second most common virulence factor among Finnish strains and present in the most frequently detected combinations; altogether 27 of the155 strains were CNF2-positive. CNF1 is far less prevalent in bovine *E. coli* strains (Burns et al., 1996). In our study, only 13 of the 155 isolates were positive for CNF1.

Other *E. coli* virulence factors connected with the survival of bacteria in the host and acting against the host defence systems have also been studied (**Table 1**). Different siderophores are important for *E. coli*, chelating iron for subsequent bacterial use. In mammary infections, this ability is thought to be more important than in other infections since milk contains lactoferrin, which also chelates iron and efficiently inhibits the growth of Gram-negative bacteria. Lin et al. (1998) found all isolates from bovine intramammary infections to be positive for siderophore production. In other studies, less than 20% have been positive for the most extensively studied siderophore, aerobactin (Linggod et al., 1987; Nemeth et al., 1994). We found only 8.4% of the isolates to be positive for aerobactin, 11% of Finnish and less than 5% of Israeli isolates (**Table 6**), suggesting that the prevalence differs geographically. Another siderophore, enterobactin, has a higher affinity for iron than aerobactin (Bagg and Neilands, 1987) and may be more important in mastitis, especially during the dry period, when the amount of iron is lower.

Serum resistance has earlier been considered an obligatory virulence factor for mastitis-causing *E. coli* bacteria (Carroll and Jasper, 1977). Some later studies have also reported serum-sensitive isolates from naturally occurring mastitis cases (Nemeth et al., 1991, 1994; Fang and Pyörälä, 1996). In our study, 41% of all isolates were serum-resistant and 16% serum-sensitive. These results show that sensitive isolates are more common than suggested earlier (Carroll and Jasper, 1977; Sanchez-Carlo et al., 1984a; Barrow and Hill, 1989; Nemeth et al., 1991, 1994; Fang and Pyörälä, 1996). According to previous studies, infections caused by serum-resistant isolates were more severe than those caused by serum-sensitive isolates (Carroll et al., 1973; Barrow and Hill, 1989). In our study, the Finnish isolates were more resistant to serum, which was unexpected, as *E. coli* mastitis in Israel is usually more severe than that seen in Finland (Shpigel et al., 1994; Sandholm and Pyörälä, 1995). Our findings make the significance of serum resistance in the pathogenesis of *E. coli* mastitis questionable. The bacteria killing ability of serum is based on the activation of the complement system. The amount of complement in milk has been speculated to be too low to cause bacterial lysis (Rainard, 2003). In *E. coli* mastitis, both the classical and alternative pathways of complement activation are important (Hogan et al., 1989). The killing ability of serum not only depends on the serum sensitivity of the isolate but also on the production of different host-derived complement factors and antibodies (Rainard, 2003).

The outer membrane protein TraT is a common virulence factor found in *E. coli* isolated from healthy and diseased animals (Sukupolvi and O´Connors, 1990; Nemeth et al., 1991). In our study,

this was the virulence factor most frequently detected, with 38% of all isolates being TraT-positive. This finding is in agreement with results reported by Nemeth et al. (1991). TraT is supposed to play an important role in the serum resistance of bacteria (Sukupolvi and O´Connors, 1990). However, in Nemeth et al. (1991), 32% of mastitis strains were TraT-positive and serum-resistant, but no association was found between TraT and serum resistance. In our study, these traits appeared to be associated, as 44.7% of serum-resistant isolates were also positive for TraT, whereas only 27.3% of serum-sensitive isolates were TraT-positive. Similar proportions were found when the Finnish and Israeli isolates were concidered separately.

All virulence factors but TraT were rare in Israeli isolates. This striking difference between the two countries is interesting. Differences between geographical areas have also been reported earlier. *E. coli* strains positive for CS31A, for instance, were found less frequently in Argentina than in the countries of the Nordic hemisphere (Mercado et al., 2003). In the Netherlands, 55% of isolates from bovine mastitis expressed F17-related fimbriae (Nemeth et al., 1994; Lipman et al., 1995), which is far more than in our study. Difference between countries or even herds may be caused by different management systems. In Finland, calves are kept together with cows, thus sharing the same pool of bacteria, whereas in Israel they are strictly separated and bacterial exchange is unlikely. *E. coli* isolates from faeces of healthy and diarrhoeic calves frequently possess virulence factors F17 and CNF2 (T. Pohjanvirta, a personal communication). Different management practices of herds may explain the differences seen in the proportions of these virulence factors between countries, Israeli *E. coli* isolates may, of course, have some other virulence factors not studied here.

The *E. coli* isolates were divided into two major groups based on composition and genetic location of their virulence factor genes. Genes for F17-related fimbriae, CNF2, Afa and TraT, which are located on plasmids (Sukupolvi and O'Connors, 1990; Falbo et al., 1992; le Bouguenec and Bertin, 1999; Mainil et al., 1999), were found together in isolates, and these strains formed one major group. The other major group contained the genes for CNF1 and S and P fimbriae, which are located chromosomally (Falbo et al., 1992; Mainil et al., 1997, 1999). These genes were always found in combination, consistent with previous reports (Bertin et al., 1998). Genes for aerobactin, located on either a chromosome or plasmid, could belong to either group.

Altogether 27% of isolates were resistant to one or more antimicrobial agents. Most common was resistance to tetracycline, which accounted for 15% of all isolates. This is still a relatively low figure compared with other published studies, in which the proportion of resistant isolates has ranged from 26% to 74% (Sogaard, 1982; Anderson, 1989; Trolldenier, 1995; Anonymous, 2001; Erskine et al., 2002a). In our study, 11% of all isolates were multiresistant. All but one multiresistance pattern included resistance to tetracycline, which is often associated with resistance to other antimicrobials (Orden et al., 2000; Österbland et al., 2000; Oppegaard et al., 2001; Tibbets et al., 2003). Several studies on *E. coli* strains of animal origin have found an association between tetracycline-, ampicillin- or multiresistance and possession of such virulence factors as enterobactin, aerobactin, colicin, F5, F41, F17, CNF, verotoxin and eae gene (Orden et al., 2000; Johnson et al., 2002; Tibbets et al., 2003). Nemeth et al. (1991) also found a positive correlation between TraT and resistance to ampicillin, streptomycin, sulphamethoxale and tetracycline in mastitis-causing *E. coli* isolates. By contrast, in a study on *E. coli* originating from septicaemic foals, aerobactin was not connected to antimicrobial resistance (Hirsh et al., 1993). In our study, resistance to tetracycline was associated with presence of virulence factors S and P fimbriae, CNF1, CNF2, aerobactin and TraT. A link was also found between ampicillin resistance and aerobactin, as well as between DHS resistance and CNF2, F17 and aerobactin. Although resistance to tetracycline was associated with the virulence genes located in chromosomes (Bertin et al., 1996), resistance is more likely to be

transmitted by plasmids and to be associated with genes located in that plasmid, such as aerobactin and CNF2.

As the genes for virulence factors partly reside in the same plasmids as the genes for antimicrobial resistance (Levy, 1992; Prescott, 2000), it is possible that the use of antimicrobials selects for certain virulence factors together with resistance to antimicrobial agents. Some studies have shown that the use of antibiotics has directly contributed to an increased prevalence of resistance (Aarestrup, 1999; Anonymous, 2001). Multiresistant bacteria also tend to maintain their resistance to a particular antimicrobial, even when that antimicrobial is absent from the environment, if the other factors to which the resistance is linked are still present (Levy, 1992; Prescott, 2000; Galland et al., 2001).

The use of antimicrobial agents in dairy herds is controlled in both Finland and Israel. Antimicrobials are used for cattle for therapeutic purposes only, and subtherapeutic or growth-promoting use is not allowed. This may be relevant to the interpretation of our results, especially since the more resistant strains of *E. coli* are suggested to originate from calves, due to their greater exposure to antibiotics (Linton et al., 1979). The controlled use of antimicrobials could be one reason for the low level of resistance found here, compared with other studies (Bishop et al., 1980; Sogaard, 1982; Anderson, 1989; Trolldenier, 1995; Erskine et al., 2002a). However, because most studies have used different methods for susceptibility testing, comparisons should be made with caution.

We found no association between virulence factors and severity of mastitis in the Finnish material. One reason for this could be that very severe cases were missing from the Finnish material. Because clinical signs and outcome of *E. coli* mastitis have been shown to be more severe in Israel compared with Finland (Shpigel et al., 1994; Pyörälä and Pyörälä, 1998), if bacterial virulence factors had a significant effect on the severity of the disease, they would have been more common among Israeli strains, which they were not. On the other hand, the Israeli cows may have more stress factors than Finnish cows due to the hotter climate, more intensive management, much bigger herd size and higher milk production. The amount of *E. coli* in the environment of the cow is probably higher in an Israeli managing system than in Finland. For these reasons, the cows in Israel could generally be at greater risk and more susceptible to *E. coli* infections. One could then hypothesize that in Israel any *E. coli* strain might cause mastitis, whereas in Finland due to perhaps more resistant cows, only the more virulent *E. coli* strains cause mastitis. Two studies have shown that the production of different virulence factors among *E. coli* strains isolated from mastitis and from faeces of healthy cows is similar (Carroll and Jasper, 1977; Nemeth et al., 1994). Mastitis-causing *E. coli* are known to originate from the environment of the cow and their virulence probably reflects the situation of bacteria found in the intestines or faeces of the cow. Based on previous studies (Carroll et al., 1973; Carroll and Jasper, 1977; Linton et al., 1979; Linton and Robinson, 1984; Sanchez-Carlo et al., 1984b; Valente et al., 1988; Barrow, 1989; Nemeth et al., 1994; Lipman et al., 1995; Jung, 1999) and our own results, we conclude that the virulence factors of the infecting bacterial strain probably play a minor role in the severity of clinical signs of *E. coli* mastitis.

However, some evidence indicates that the outcome of *E. coli* mastitis may be related to some characteristics of the infecting bacterial strain. Previous studies have suggested the existence of udder-adapted *E. coli* strains, which could produce chronic or recurrent *E. coli* mastitis (Lipman et al., 1995; Lam et al., 1996a; Döpfer et al., 1999; Bradley and Green, 2001). Recurrent cases tended to be milder than non-recurrent cases, which could favour the persistence of bacteria in the udder (Bradley and Green, 2001). In our study, presence of genes for S and P fimbriae was significantly

associated with persistence of mastitis. In humans, *E. coli* with S and P fimbriae is associated with recurrent cases of urinary tract infections (Schilling et al., 2001).

Definition of persistent infection in recurrent *E. coli* mastitis is difficult. Fingerprinting techniques are useful to identify the strains and our study would have benefited from the use of those techniques. Same *E. coli* genotypes have been found in recurrent cases of mastitis, but in some studies 63% were of different genotypes (Döpfer et al., 1999) or serotypes (Linton and Robinson, 1984). However, even if identical strains would be detected, they could be those predominating in the cow's environment, and the possibility of reinfection cannot be excluded. In addition, in earlier research as well as in our study, the cases were not followed up throughout the study period, which would be necessary to confirm the real persistence of infection.

Host response has a major role in coliform mastitis, and the clinical course and final outcome of the disease is mostly based on the reactions of the host, the cow. Different physiological factors have a substantial effect, the most important being parity and lactation stage of the cow. We did not study the effect of cows' age on host response, but evidence from earlier studies has shown that young cows are more resistant than older cows due to their more alert defence mechanisms (van Werven et al., 1997; Mehrzad et al., 2002b).

Lactation stage greatly affects the susceptibility of the cow to *E. coli* infections, and early-lactating cows are the most susceptible (Burvenich et al., 2003; Hogan and Smith, 2003). During the dry period, when the lactoferrin concentration in milk is high, clinical *E. coli* mastitis seldom occurs. Some *E. coli* strains can, however, produce siderophores to survive in a low iron environment (Lin et al., 1998, 1999) and therefore may infect the cow (Bradley and Green, 2000). If *E. coli* mastitis is detected during the dry period, it usually occurs 1-2 weeks before calving, when the udder is no longer fully involuted and colostrogenesis has started (Smith et al., 1985; Bradley and Green, 2000). In a fully involuted udder, spontaneous coliform mastitis is rare (Smith et al., 1985), and induction of experimental *E. coli* infection has failed (Todhunter et al., 1990).

Cows are known to be most susceptible to coliform mastitis during the puerperial period (Kehrli et al., 1989; Kremer et al., 1990; Shuster et al., 1996; Mehrzad et al., 2002b; Hogan and Smith, 2003). There are several reasons for this phenomenon; at this stage, the metabolic state of the cow does not favour efficient defence, and stress around parturition, which is related to high cortisol levels, interferes with cellular defence (Breazile, 1988). Our field data showed an increased incidence during the post-partum period, as *E. coli* mastitis was most common in EL (0-21 days PP), decreasing towards the dry period.

During the puerperial period and EL, cows suffering from mastitis due to *E. coli* have more severe clinical signs than cows in LL (Hill et al., 1979; Pyörälä and Pyörälä, 1998; Mehrzad et al., 2002b). This was also seen in our experimental endotoxin study. However, in our field data, the clinical signs unexpectedly tended to be milder in EL (0-21 days PP) than in LL. This may reflect the difficulty of defining severity in spontaneous mastitis. In the field, mastitis may be diagnosed by the herd owner about 12 h after onset of infection (Lohuis et al., 1990b; Hirvonen et al., 1999; Hoeben, 1999; Haddad et al., 2001). If veterinary consultation is sought, the diagnosis is made several hours later. Assessment of the severity of spontaneous coliform mastitis in practice occurs relatively late in the course of the disease, after, for example, the fever peak has already passed. This may be one reason for the milder clinical signs often reported in naturally occurring coliform mastitis than in experimental studies (Haddad et al., 2001).

The severity of the response of cows in *E. coli* mastitis varies markedly between individuals (Vandeputte-Van Messom et al., 1993; Hirvonen et al., 1999). One explanation for this individual variation may be the different sensitivity of cows to environmental stressors, as increased stress and rise of plasma cortisol are associated with a decreased number of circulating lymphocytes (Hopster et al., 1998). In our endotoxin experiment, the same cows were used in the studies during EL and LL to minimize the effect of individual variation and to determine the actual impact of lactation period. The cortisol levels of the cows in EL and LL did not differ significantly. While a cow-to-cow variation was clearly seen, the difference between EL and LL was greater than the difference between individual cows.

The ability of the immune system of the cow to resist bacterial invasion is critical in determining cows' the susceptibility to *E. coli* mastitis. Efficacy of host defence also defines how severe the course of mastitis will be. Although the host response is an extremely complicated network of different actions and components, the number and function of PMN in blood and especially in milk are critical (Burvenich et al., 2003). The initial number of PMN in blood and milk have been shown to correlate negatively with the severity of *E. coli* mastitis (Vandeputte-Van Messom et al., 1993; Shuster et al., 1996; Dosogne et al., 1997). In healthy cows, the number of PMN is lower in EL than in LL (Kehrli et al., 1989; Kulberg et al., 2002). A slightly lower initial number of blood PMN and milk SCC in EL than in LL were also seen in our study. A low number of PMN in milk may cause inefficient killing of bacteria, thus allowing them to rapidly multiply (Shuster et al., 1996). Simultaneously with an increase in milk SCC at 4 h PC, the number of circulating PMN in blood decreased (**Figures 6 and 7**), a phenomenon well known from other studies (Hoeben, 1999; Bannerman et al., 2003). Blood neutropenia caused by the migration of PMN to the infection site of the udder (Paape et al., 1974) is resolved by releasing both mature and immature neutrophils from bone marrow (Hoeben, 1999). In our study, PMN numbers in milk and blood increased faster and reached higher levels in EL cows than in LL cows. This could be due to a greater PMN recruitment during EL, as has earlier been shown in experimental *E. coli* mastitis (Shuster et al., 1996). The number of PMN in blood and milk thus may not be the reason for the increased susceptibility of EL cows to *E. coli* mastitis. PMN function may be more critical to host defence than their actual number (Lohuis et al., 1990a; Cai et al., 1994; Cooray and Håkansson, 1995; Mehrzad, 2002a). In experimental *E. coli* infection, bacteria multiplied at a significantly higher rate in milk of cows with low PMN respiratory burst activity (Vandeputte-van Messom et al., 1993; Mehrzad, 2002a). PMN function and respiratory burst activity are down-regulated by the onset of apoptosis (van Oostveld et al., 2002). This could be one reason for the decreased PMN functions seen in the peripartum period since during this time the rate of apoptosis is increased (van Oostveld et al., 2001). If the production of ROS is not adequately regulated, the respiratory burst and ROS production may become harmful to the host, resulting in tissue damage and increasing the severity of clinical response (Burvenich et al., 2003). In the puerperal period and EL, PMN ROS production may be extracellular, causing inadequate bacterial killing and more damage to the udder tissue (Mehrzad, 2002a; Burvenich et al., 2003).

Function of milk and blood PMN measured as CL has been reported to be significantly lower in EL than in LL in healthy cows (Hoeben et al., 2000b; Mehrzad et al., 2001b). A lower preinfection respiratory burst was associated to lower glucose, magnesium, calcium and cortisol levels and higher FFA levels in blood of cows (Vandeputte-van Messom et al., 1993). ROS production of blood PMN was lower in cows with higher fat infiltration in the liver at 7-10 days PP (Zerbe et al., 2000). In our study, no difference was found in preinfection CL levels between EL and LL cows. This could be due to the different methods used; we used zymosan-induced CL, which also measures phagocytosis and not only respiratory burst, as does PMA-induced CL, which was used in the studies of the other authors (Hoeben et al., 2000b; Mehrzad et al., 2001b). Phagocytosis of PMN

is known not to be depressed in EL (Dosogne, 1998). We found that the initial CL was lower in milk cells than in blood cells, a finding also reported earlier (Mehrzad et al., 2001a). The lower CL of milk PMN after zymosan induction may be caused by prestimulation of milk PMN by the milk components and exhaustion of the cells (Paape et al., 2003). Phagocytosis may also be impaired due to the limited energy reserves in milk (Paape et al., 2003).

After the endotoxin challenge, the function of blood neutrophils measured as CL increased in LL but decreased in EL. The reason for the decreased CL after parturition and during *E. coli* infections has been suggested to be due to the increased proportion of immature neutrophils in blood (Dosogne, 1998). Immature neutrophils have been shown to have an impaired respiratory burst ability (Glasser and Fiederlein, 1987). In our study, a low CL was seen simultaneously with neutropenia, when immature neutrophils are not yet released. In addition, as mentioned earlier, zymosan-induced CL measures not only respiratory burst of PMN but also their capacity for phagocytosis (Hoeben, 1999), which is already present at an early stage of PMN maturation (Glasser and Fiederlein, 1987). One explanation for the impaired CL in blood PMN could be subclinical ketosis of the cow (Hoeben et al., 1997; Suriyasathaporn et al., 2000; Zerbe et al., 2000), reflected by increased serum FFA concentrations in EL cows (Burvenich et al., 1994; Jorritsma et al., 2001; Nikolic et al., 2003), as also seen in our study.

The CL of milk PMN increased in both EL and LL after the endotoxin challenge. The increase in CL of milk during both lactation periods appeared at the same time as SCC increased. The increase was more pronounced in EL, appearing simultaneously with the decreased CL of PMN in blood. This may result from an influx of the most active PMN from the circulation to the milk (Dosogne, 1998). The milk cells achieved a higher CL than blood neutrophils, supporting the finding of Mehrzad et al. (2001a). This could be due to the local production of proinflammatory mediator TNFα and other cytokines that trigger PMN functions (Paape et al., 2003).

TNFα was detected in the milk, but not in the serum of cows in both EL and LL. In previous studies, TNFα has been detected in the plasma and milk of early-lactating cows (Blum et al., 2000; Hoeben et al., 2000a) but not in cows in mid-lactation (Paape et al., 2002). Levels of TNFα have been much higher in milk than in serum (Sordillo and Peel, 1992; Nakajima et al., 1997; Hirvonen et al., 1999; Blum et al., 2000; Hoeben et al., 2000a; Hisaeda et al., 2001; Ohtsuka et al., 2001; Paape et al., 2002). In the milk of our cows, TNFα concentration was higher in EL cows, although the difference was not significant because of the great variation between individuals (**Figure 6**). Increased TNFα production around the time of calving has also been found by others. Periparturient dairy cows have been reported to have an increased number of monocytes and macrophages with increased expression of the membrane receptor mCD14, which may enhance the capacity of these cells to secrete TNFα *in vitro* (Sordillo et al., 1995; Røntved et al., 2004). EL and LL cows may also have differences in the amount of soluble sCD14 receptors. An increased amount of sCD14 decreases the amount of TNFα because it binds the LPS-LBP complex instead of mCD14, which otherwise would lead to release of TNFα from macrophages (Lee et al., 2003; Wang et al., 2003). Recruitment of neutrophils increases, leading to a faster clearance of bacteria, and thus, milder clinical signs (Lee et al., 2003).

We found TNFα production to peak at 8 h PC, when SCC had also reached its highest level. This is in accordance with previous evidence that milk cells are the main source of TNFα, although during inflammation mammary epithelial cells may also produce some TNFα (Pfaffl et al., 2003). The TNFα concentration in milk does not increase before the manifestation of clinical signs but is detected at the same time as the systemic signs and later than the local signs (Blum et al., 2000; Hoeben et al., 2000a; Lee et al., 2003). After intramammary and intraveous infusion of endotoxin,

the concentration of TNFα in the blood was found to increase later than body temperature or many of the metabolic changes, indicating that these signs are not mediated by circulating TNFα (Werling et al., 1996; Steiger et al., 1999; Hoeben et al., 2000a). Although TNFα production has generally been shown to peak in milk simultaneously or later than clinical signs, small amounts of TNFα have been detected shortly after endotoxin challenge (Persson Waller et al., 1997; Hoeben et al., 2000a). TNFα appears to have a critical role in initiating host response by inducing the local production of IL-1, IL-6 and IL-8 (Watanabe et al., 2000; Paape et al., 2002; Wang et al., 2003), which in turn mediate the systemic effects of the host response (Van Miert, 1991; Shuster et al., 1993). TNFα also has other roles in the local reaction. *In vitro* studies have shown the stimulating effect of bovine recombinant TNFα on neutrophil functions (Chiang et al., 1991; Sample and Czuprynski, 1991). Locally active TNFα and IL-1 may also activate the expression and action of matrix metalloproteinase (**MMP**) (Hanemaaljes et al., 1997). The metalloproteinases MMP_2 and MMP_9 were found to peak at 2-4 h after endotoxin challenge, 2 h before the SCC increase in milk, and were suggested to enhance vascular permeability, enabling the invasion of PMN into the milk (Li et al., 1999; Raulo et al., 2002). TNFα has usually only been detected in blood of cows with very severe mastitis (Pedersen et al., 2003). The positive correlation between severity of host reaction and concentration of TNFα in blood or milk seen in previous studies (Hirvonen et al., 1999; Blum et al., 2000; Hisaeda et al., 2001; Ohtsuka et al., 2001) may be due to the abnormal regulation of a normal TNFα-mediated response (Burvenich et al., 2003). The effect of TNFα may become deleterious to the host when its production is excessively high (Lee et al., 2003) or when production does not cease sufficiently early.

Production of acute-phase proteins, such as SAA and haptoglobin, at a later stage of the host response is related to the repair processes after tissue damage, detoxification of LPS (Urieli-Shoval et al., 2000) and down-regulation of pro-inflammatory responses (Uhlar and Whitehead, 1999). In our study, SAA increased in both serum and milk after alleviation of clinical signs. When clinical signs were the most intense, the SAA concentration in milk had reached only one-fourth of its highest value. These early-phase concentrations of SAA were similar to those found by Eckersall et al. (2001) in spontaneous clinical mastitis. SAA peaked earlier in milk than in serum, but the levels of SAA in serum did not follow those of milk. This supports the idea that SAA must be produced and regulated both locally and systemically (Eckersall et al., 2001; McDonald et al., 2001). TNFα was not found in serum which indicates that in cattle systemic SAA production is not induced by TNFα but by some other cytokines, such as IL-6, which increase in the serum after LPS stimulation or during *E. coli* mastitis (Shuster et al., 1993; Werling et al., 1996; Nakajima et al., 1997). The cytokine IL-6 has been shown to be a potent inducer of hepatic production of SAA (Alsemgeest et al., 1996). In milk, the concentrations of TNFα and SAA seemed to be closely related; cows with very low TNFα levels also had low SAA concentrations, and those with high TNFα levels had high SAA concentrations. A positive correlation between TNFα and SAA has also been observed in human patients with sepsis (Uhlar and Whitehead, 1999). Locally produced SAA may also induce the production of TNFα and other cytokines by neutrophils (Furlaneto and Campa, 2000).

TNFα and SAA concentrations in milk and serum have been suggested to correlate with the severity of the cow's response (Sordillo et al., 1995; Hirvonen et al., 1999; Blum et al., 2000; Eckersall et al., 2001; Hisaeda et al., 2001; Ohtsuka et al., 2001). Changes in milk appearance during mastitis are partly caused by loosened cell connections in the udder epithelia, allowing passage of proteins, enzymes, cells and trace elements between milk and blood. Thus, because of the increased permeability between milk and blood compartments of the udder, some of the SAA detected in the serum may originate from milk. This may also explain the trace amounts of TNFα detected in serum in some studies. Here, more severe systemic and local signs were seen in EL cows, which also had higher average concentrations of TNFα and SAA in milk. SAA concentrations in serum, by

contrast, were lower in EL than in LL. One reason for this could be the compromised function of hepatocytes during the peripartum period due to a fatty liver, resulting in decreased production of SAA.

The results here support previous observations that increased susceptibility of the cow to *E. coli* mastitis and more severe disease course during EL depend more on host factors than on bacterial properties. The virulence and antimicrobial resistance of mastitis-causing *E. coli* reflect the situation of bacteria in the environment or in the intestines or faeces of the cow. To prevent of *E. coli* mastitis, more efforts should be directed at improving the cow's environment and herd management. Although the literature on bovine *E. coli* mastitis is abundant, knowledge on its pathogenesis remains far from complete. Further studies should focus on host response and other cow factors related in this complex disease. Especially cytokine and interleukin interaction at the early stage of host response as well as the role of APP in the inflammation process should be investigated. One advantage of *E. coli* mastitis research is that good infection and inflammation challenge models are available; our cross-over study design diminishes the cow-to-cow variation. Field studies on spontaneous mastitis are also necessary to confirm findings of experimental research. For improved comparisons between different studies, the definitions of clinical *E. coli* mastitis characteristics (mild, moderate, severe, persistent) should be standardized. A deeper understanding of *E. coli* mastitis pathogenesis may help us to improve the immune defence of dairy cows against coliform mastitis and to develop more effective treatments.

10. CONCLUSIONS

1. *E. coli* isolates from clinical bovine mastitis in Finland and Israel possessed a variety of different virulence factors, but none of these was common. To infect the bovine udder, specific virulence factors do not seem to be necessary for *E. coli* bacteria.

2. The differences in prevalence of virulence factors and in clinical signs of *E. coli* mastitis in both Finland and Israel suggest that the virulence factors of the bacterial strain probably play a minor role in the severity of *E. coli* mastitis.

3. The presence of genes for S and P fimbriae, CNF1 and CNF2 was significantly associated with persistence of mastitis. Recurrent *E. coli* mastitis could be caused by udder-adapted *E. coli* strains, and the outcome from *E. coli* mastitis may be related to certain characteristics of the bacterial strain.

4. Antimicrobial resistance of *E. coli* isolates was low; 27% of the isolates were resistant to one or more antimicrobial agents and 11% were multiresistant. All but one multiresistance pattern included resistance to tetracycline. Associations between tetracycline resistance and S and P fimbriae, CNF1, CNF2, aerobactin and TraT, ampicillin resistance and aerobactin, and DHS resistance and CNF2, F17 and aerobactin were found.

5. In the experimental endotoxin mastitis, cows showed more severe systemic signs in EL than in LL.

6. TNFα concentration in milk increased after the endotoxin challenge, reaching higher levels in EL. In blood, no TNFα was detected. TNFα may not be directly responsible for the systemic signs in endotoxin mastitis, but it probably has a critical role in initiating the host response. TNFα also seems to induce production of SAA in milk since the concentration of SAA in milk was higher in EL than in LL, and the concentrations of TNFα and SAA were closely related at the cow level. In blood, SAA increased later than in milk after endotoxin challenge, and the average concentration was higher in LL, indicating differences in production and regulation of local and systemic SAA.

7. After induction of endotoxin mastitis, PMN numbers in milk and blood increased faster and reached higher levels in EL than in LL. However, the function of blood neutrophils, measured as CL, increased in LL but decreased in EL after the endotoxin challenge. This impaired function of blood PMN in EL could be caused by subclinical ketosis of the cow, reflected in increased serum FFA concentrations. The CL of milk PMN increased in both EL and LL, being more pronounced in EL and appearing simultaneously with the decreased CL of PMN in blood. This could have resulted from an influx of the most active PMN from the circulation to the milk, and from the stimulation of milk PMN by locally produced cytokines such as TNFα. The function of PMN may therefore be more critical for host defence and for susceptibility of cows to *E. coli* mastitis than their actual number in blood and milk.

11. ACKNOWLEDGEMENTS

This study was carried out at the Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki. Financial support provided by the Finnish Academy of Science, the Finnish Veterinary Foundation, the Research Foundation of Veterinary Medicine, the Walter Ehrström Foundation, and Valio Ltd., is gratefully acknowledged.

I particularly would like to thank the late Juhani Hirvonen, DVM, without whom I never would have considered engaging in any scientific project. I initially had no plans for academic career or PhD, instead my goal was to become a practitioner in the countryside. But after seeing how Juhani, a great scientist, was able to use his scientific knowledge in practice and become though even a better practitioner, I was encouraged to take part of this research project on *E. coli* mastitis in dairy cows.

Another reason for becoming involved in this particular project was that it was supervised by Professor Satu Pyörälä who I greatly admired. After these years, I still do, only much more, and I am very grateful for her endless support and guidance during my PhD project and also for becoming a good friend.

I am indebted to my other supervisor Professor Sinikka Pelkonen for her support, and for excellent guidance in the molecular biological parts of the work. I am grateful to my co-authors Tarja Pohjanvirta, DVM from the National Veterinary and Food Research Institute, Kuopio and Professor Tuula Honkanen-Buzalski, from the National Veterinary and Food Research Institute, Helsinki for their guidance in scientific work and microbiological laboratory analyses, to Christina Røntved, DVM for the TNFα analyses, to Sami Suominen, DVM and Taina Kutila, DVM, PhD for their help in experimental mastitis study, and to Adin Shwimmer, DVM and Professor Nahum Shpigel for providing me material from Israeli cows. To Professor Nahum Shpigel I am also grateful for the opportunity to do part of my research in Israel, in the University of Jerusalem, under his supervision.

The pre-examiners of the dissertation, Professor Christian Burvenich and Andrew Bradley, MA, VetMB, DCHP, DiplECBHM, PhD, MRCVS are thanked for their constructive criticism at the final stage of this work. Professor Karin Persson Waller is thanked for agreeing to serve as my opponent.

I am grateful to Satu Sankari, DVM, PhD, Marja-Liisa Tasanko and Hannu Sipilä for skilful assistance in laboratory analyses, to Hannele Pentti and Reijo Jokivuori for their assistance in experiment arrangements, to Arto Ketola, Msci (Soc.) and Samuli Ripatti, PhD for help in statistical analysis, and Caroll Ann Pelli, HonBSc, for her valuable language revision of my articles and manuscript.

My warmest gratitude goes to all my friends and colleagues, especially former ones in Saari Unit, members of the “dynamic mastitis group”, and my present colleagues in Sisä-Savo for friendship and support and all those inspiring discussions of mastitis, cows and veterinary medicine in general.

Finally, I present my deepest gratitude to my beloved husband Tuukka for his love, which is the most precious to me.

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